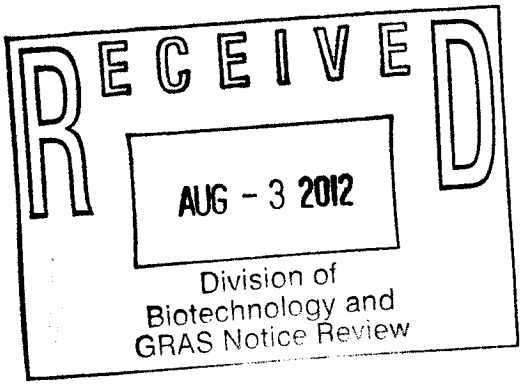
ORIGINAL SUBMISSION

Ester-C®
The Ester C Company



SENT *VIA* FEDEX

July 25, 2012

Mary Ditto, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for Ester-C®

Dear Dr. Ditto:

In accordance with 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting in triplicate, as the Notifier [The Ester C Company, 6735 Inter-Cal Way Prescott, Arizona, 86301, USA], a Notice of the determination, on the basis of scientific procedures, that Ester-C® [calcium ascorbate with a content of threonate] distributed by The Ester-C company, as defined in the enclosed documents, is GRAS under specific conditions of use as an ingredient in multiple food categories, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, includes a comprehensive summary of the data available that has been reviewed by an independent panel of experts (the Expert Panel) qualified by scientific training and experience to evaluate the safety of Ester-C in traditional food products.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

Maile Combs, MS
Assoc. Director Scientific Affairs
The Ester C Company

000002

GRAS Exemption Claim for Ester-C® Calcium Ascorbate (Ester-C®)

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
(CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Submitted by:

The Ester C Company
6735 Inter-Cal Way
Prescott, Arizona 86301
USA

July 12, 2012

GRAS Exemption Claim for Ester-C® Calcium Ascorbate (Ester-C®)

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List of Figures and Tables

I GRAS Exemption Claim

I.A Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)] (U.S. FDA, 1997)

We hereby claim that the use of Ester-C® [calcium ascorbate with added threonate] as a nutrient in foods, as described in Section I.D below, is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because we have determined that such use are Generally Recognized as Safe (GRAS).

Signed,

Maile Combs MS
Assoc. Director Scientific Affairs
The Ester C Company

Date

I.B Name and Address of Notifier

The Ester C Company
6735 Inter-Cal Way
Prescott, Arizona 86301
USA
Tel: 928-541-2269
Fax: 928-777-2459

I.C Common Name of the Notified Substance

Calcium ascorbate with threonate

I.D Conditions of Intended Use in Food

1. Foods in Which the Substance is to be Used

The Ester C Company intends to use Ester-C® as a nutrient in conventional foods and beverages. The individual intended food uses, maximum use-levels for Ester-C®, and corresponding maximum use-levels of ascorbic acid (vitamin C), are summarized in Table I.D-1. In those foods and beverages already fortified with a source of vitamin C, Ester-C® is intended to be added in replacement of, and not in addition to, the existing vitamin C fortificant. In addition to the traditional food uses listed in Table I.D-1, the Ester C Company also intends to

use Ester-C® as a nutrient in medical foods at use-levels providing up to 500 mg of vitamin C per serving.

Table I.D-1 Summary of the Individual Intended Food Uses and Use-levels for Ester-C® and the Corresponding Use-levels of Vitamin C in the U.S.

Food Category	Intended Food Uses	RACC (g/mL)[a]	Ester-C®		Vitamin C	
			Use level[b] (mg/ serving)	Use level (%)	Level (mg/ serving)	Level (%)
Beverages and Beverage Bases	Energy Drinks	240	326	0.14	250	0.10
	Fruit-Flavored Drinks and Ades (with Vitamin C Added)	240	326	0.14	250	0.10
	Meal Replacement Beverages (Powdered Only)	240	326	0.14	250	0.10
Breakfast Cereals	Instant Oatmeal	240	78	0.033	60	0.025
	RTE Breakfast Cereals[c]	15 (Puffed) 30 (Regular) 55 (Biscuit-Type)	78	0.52 0.26 0.14	60	0.40 0.20 0.11
Chewing Gum	Chewing Gum	3	163	5.44	125	4.17
Coffee and Tea	RTD Teas (Presweetened, Not Powdered)	240	163	0.068	125	0.052
Grain Products	Breakfast and Meal Replacement Bars	40	163	0.41	125	0.31
Processed Fruits and Fruit Juices	Fruit Juice (Excluding Fruit Juice Blends)[c]	240	326	0.14	250	0.10
Medical Foods	Medical Foods	N/A	653	N/A	500	N/A

N/A = not applicable; RTD = Ready-to-drink; RTE = Ready-to-eat
[a] RACC = Reference Amounts Customarily Consumed per Eating Occasion (21 CFR §101.12 – U.S. FDA, 2012a). When a range of values is reported for an intended food use, particular foods within that food use may differ with respect to their RACC.
[b] Use level of Ester-C® = Use level of Vitamin C/0.766
[c] The food codes listed under this food use were selected by The Ester C Company.

2. Purpose for Which Substance is Used

Ester C is intended to be used in foods as a source of the nutrient ascorbic acid.

3. Description of the Population Expected to Consume the Substance

Ester C is expected to be consumed by members of the general population who may be reasonably be expected to consume at least one food within the food categories described above.

I.E Basis for the GRAS Determination

Pursuant to Title 21, Section 170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2012b), Ester-C® [calcium ascorbate with threonate] has been determined to be GRAS on the basis of scientific procedures.

I.F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

The Ester C Company
6735 Inter-Cal Way
Prescott, Arizona 86301
USA

Should the FDA have any questions or additional information requests regarding this notification, the Ester C Company will supply these data and information.

II Detailed Information about the Identity of the Notified Substance

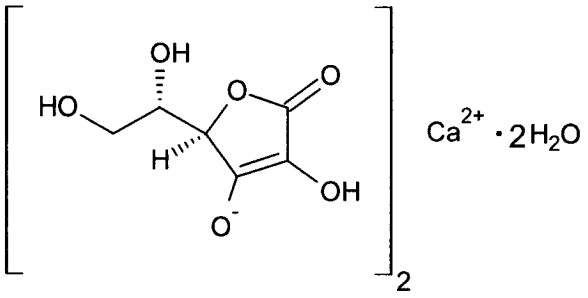
II.A Identity

Ester-C® is a tan, free flowing powder with a slight caramel odor and is characterized as comprising 95.5% calcium ascorbate dihydrate, 1.2% calcium threonate, 1.1% calcium carbonate and not more than 2.0% moisture by weight. Ester-C® is free of genetically modified organisms, contains no materials of animal origin, is free from artificial colors, flavors, and preservatives, is not irradiated or sterilized in any way, and does not contain any carriers.

Common or Usual Name:	Calcium ascorbate
Chemical Name:	Calcium ascorbate dihydrate
Chemical Abstracts Service (CAS) Number:	201542-81-6
Empirical Formula and Formula Weight:	$C_{12}H_{14}CaO_{12}\cdot 2H_2O$

Chemical Structure:

Figure II.A-1 Calcium Ascorbate Dihydrate



Common or Usual Name: Calcium threonate

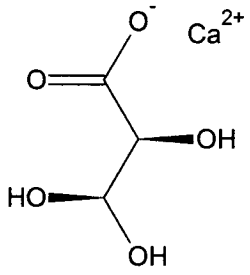
Chemical Name: Calcium L-threonate

Chemical Abstracts Service (CAS) Number: 70753-61-6

Empirical Formula and Formula Weight: $CaO_2C_3H_4$

Chemical Structure:

Figure II.A-2 Calcium L-Threonate



1. Impurities

During the manufacturing of calcium ascorbate, small quantities of hydroxymethyl furanone are produced as a byproduct of the thermal reaction process. The quantities of hydroxymethylfuranone produced during manufacturing are limited to 0.045 mg/100 g in the finished product.

Common or Usual Name: hydroxymethyl furanone

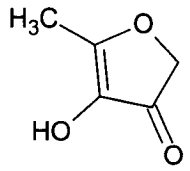
Chemical Name: 4-hydroxy-5-(methyl-3(2H)-furanone

Chemical Abstracts Service (CAS) Number: 19322-27-1

Empirical Formula and Formula Weight: $C_5H_6O_3$

Chemical Structure:

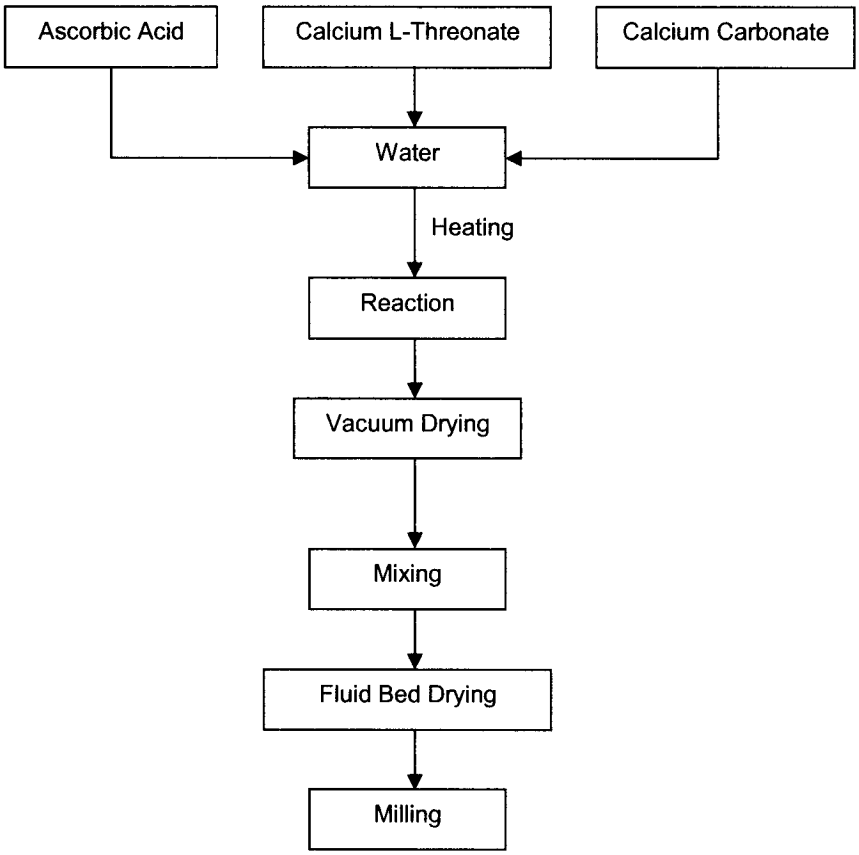
Figure II.A-3 Hydroxymethyl Furanone



II.B Method of Manufacture

A schematic diagram of the manufacturing process employed to produce Ester-C® is illustrated in Figure II.B-1. Ester-C® is produced in bulk with precise temperature control using a water-based method. Ascorbic acid is first dissolved in water at 140°F in an industrial mixer. Calcium L-threonate is added intermittently to the solution, which is neutralized with the addition of calcium carbonate. The reaction is then carried out under steam pressure (>100°F), and followed by vacuum drying. The partially dry contents are then transferred to a holding vessel, and held with mixing by use of an industrial ribbon blender at room temperature. The material is dried to completion using a fluid bed dryer and then milled into granular form or fine powder with the use of an industrial mill and the appropriate screen. A purification step is not included and no processing aids are used.

Figure II.B-1 Schematic Overview of the Manufacturing Process for Ester-C® Calcium Ascorbate



II.C Specifications for Food Grade Material

Chemical and physical specifications for Ester-C® are presented in Table II.C-1. Ester-C® comprises 76.60 mg/100 mg ascorbic acid, 9.18 mg/100 mg calcium, and 1.15 mg/100 mg threonic acid, and hydroxymethyl furanone occurs in an amount of 0.045 mg/100 mg. Moisture content accounts for less than 2% of the final product composition. Microbiological limits for *Staphyloccoccus aureus*, *Escherichia coli*, and *Salmonella* sp. are included in the product specifications. Product specifications for Ester-C® are the same irrespective of the form, either granular or fine powder.

Analyses of 5 non-consecutive lots of Ester-C® confirm that the manufacturing process produces a consistent product (see Table II.C-2). The analytical results obtained for ascorbic acid, calcium, threonic acid, and hydroxymethyl furanone were confirmed to lie within range of the accuracy of the methods employed. To define the composition of Ester-C® to 100%, residual carbonate also was analyzed and waters of hydration were calculated. In addition, analysis of the Ester-C® ingredient for lead and microbial contaminants indicate conformance to

the limits set by the product specifications. Ester-C® is free of genetically modified organisms, contains no materials of animal origin, is free from artificial colors, flavors, and preservatives, is not irradiated or sterilized, and does not contain any carriers.

Table II.C-1 Specifications for Ester-C® Calcium Ascorbate

Specification Parameter	Specification	Method
Appearance	Free flowing powder	Organoleptic
Color	Tan	Organoleptic
Odor	Slight caramel	Organoleptic
Ascorbic acid	76.60 ± 2.4 mg/100 mg	NIR spectroscopy[a]
Calcium	9.18 ± 1.13 mg/100 mg	ICPMS[b]
Threonic acid	1.15 ± 0.30 mg/100 mg	HPLC[a]
Hydroxymethyl furanone	0.045 ± 0.015 mg/100 mg	HPLC[a]
pH	6.90 ± 0.40	pH meter[a]
Free moisture (loss on drying)	NMT 2.00%	Halogen moisture balance[c]
Lead	NMT 1 ppm	ICPMS[b]
Total aerobic microbial count	NMT 1,000 CFU/g	USP <2021>[d]
Total yeast and mold count	NMT 100 CFU/g	USP <2021>
Staphylococcus aureus	Negative	USP <2021>
Escherichia coli	Negative	USP <2021>
Salmonella species	Negative	USP <2021>

CFU = colony-forming units; HPLC = high performance liquid chromatography; ICPMS = inductively coupled plasma mass spectrometry; NIR = near infrared; NMT = not more than; USP = United States Pharmacopeia
[a] FCC (2008).
[b] JECFA (2006a).
[c] Ester C Company in-house method.
[d] USP-NF (2011).

Table II.C-2 Summary of the Chemical Product Analysis for 5 Non-Consecutive Lots of Ester-C® Calcium Ascorbate

Specification Parameter	Specification	Manufacturing Lot				
		192077[a]	193099[b]	193105[c]	195876[d]	196269[e]
Appearance	Free flowing powder	Conforms	Conforms	Conforms	Conforms	Conforms
Color	Tan	Conforms	Conforms	Conforms	Conforms	Conforms
Odor	Slight caramel	Conforms	Conforms	Conforms	Conforms	Conforms
Ascorbic acid (mg/100 mg)	76.60 ± 2.43	78.1	78.1	78.2	78.2	78.1
Calcium (mg/100 mg)	9.18 ± 1.13	8.83	9.17	8.79	9.04	8.88
Threonic acid (mg/100 mg)	1.15 ± 0.30	1.35	1.40	1.27	1.26	1.30
Hydroxymethyl furanone (mg/100 mg)	0.045 ± 0.015	0.037	0.041	0.036	0.033	0.036
Free moisture (loss on drying) (%)	NMT 2.00	1.66	1.41	1.52	1.66	1.07
Residual Carbonate (%)	Report	1.652	1.807	1.858	1.497	1.187
Waters of Hydration[f] (%)	Report	7.96	7.96	7.99	7.99	7.96
Total Composition[g] (%)	Report	99.59	99.89	99.66	99.68	98.53
pH	6.90 ± 0.40	7.00	7.18	7.01	6.75	7.02
Lead (ppm)	NMT 1 ppm	0.10	0.17	<0.05	0.07	0.08
Total aerobic microbial count (CFU/g)	NMT 1,000	<10	<10	<10	<10	<10
Total yeast and mold count (CFU/g)	NMT 100	<10	<10	<10	<10	<10
Staphylococcus aureus	Negative	Negative	Negative	Negative	Negative	Negative
Escherichia coli	Negative	Negative	Negative	Negative	Negative	Negative
Salmonella species	Negative	Negative	Negative	Negative	Negative	Negative

CFU = colony-forming units; NMT = not more than
[a] Production date: 2008/08/28
[b] Production date: 2008/10/07
[c] Production date: 2008/09/30
[d] Production date: 2008/10/08
[e] Production date: 2008/10/17
[f] Calculated from ascorbic acid assay based on 1 mole H2O per mole ascorbic acid in the Calcium Ascorbate Dihydrate molecule.
[g] By calculation

II.D Stability

The stability of the bulk Ester-C® ingredient under the intended storage conditions, as well as the ingredient under the intended uses, has been assessed, and the results are described in Sections II.D.1 and II.D.2, respectively.

1. Stability of the Bulk Ingredient

1.1 Room Temperature Study

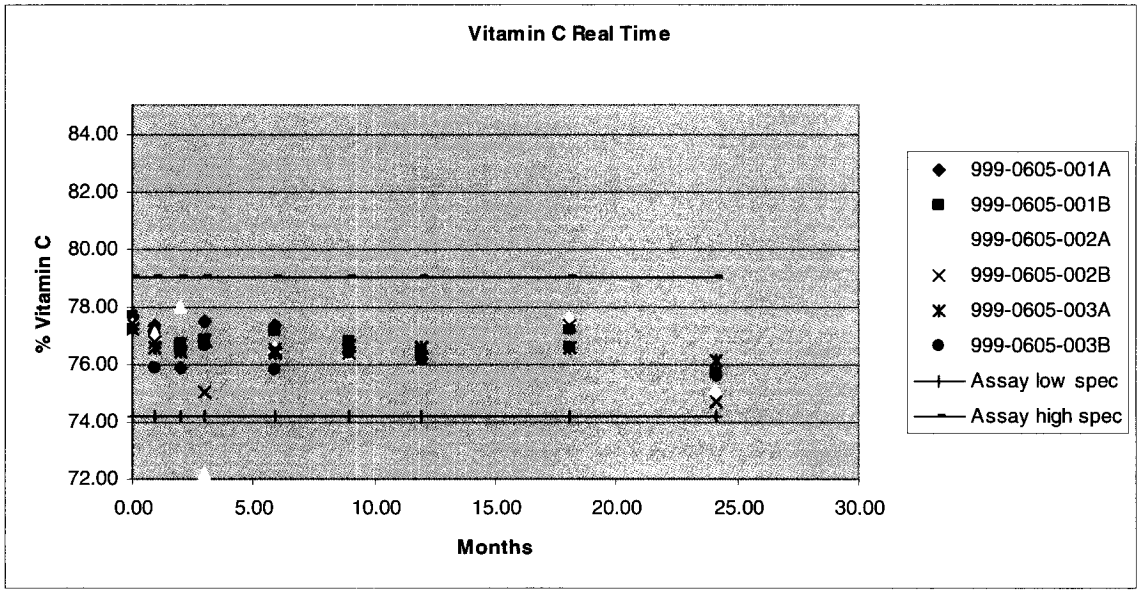
In order to evaluate the stability of the bulk ingredient under the intended storage conditions, The Ester C Company assessed 3 lots of Ester-C® (Lot Nos. 999-0605-001, 999-0605-002, 999-0605-003) under controlled room temperature conditions (25°C; 60% relative humidity) over a period of 24 months at regular intervals. Duplicate samples (A and B) from each lot at each time point were assessed for ascorbic acid, threonic acid, hydroxymethyl furanone, and free moisture content as well as for pH. The analyses indicated that the composition of Ester-C® did not vary significantly over the duration of the study and were within range of the established limits of each of the product specifications at the end of the study period. At 2 and 3 months, the hydroxymethyl furanone content was slightly above specification in 1 and 2 samples, respectively, but had decreased to within specification values for the remainder of the study. The hydroxymethyl furanone content of all other samples were within the specification range for the entire duration of the study. The results of this study are presented in Table II.D-1 and those for ascorbic acid also are presented in Figure II.D-1.

Table II.D-1 Stability of Ester-C® Calcium Ascorbate Over a 24-Month Period

Storage Term (months)	Content of Ester-C® Calcium Ascorbate Constituents[a]				
	Ascorbic acid (%)	Threonic acid[b] (%)	Hydroxymethyl furanone (%)	Free moisture (%)	pH
0.00	77.42	1.17	0.04	1.21	6.94
1	76.78	Not tested	0.05	1.15	7.04
2	76.68	1.15	0.06	1.19	7.00
3	75.82	1.12	0.06	1.28	7.00
6	76.64	1.15	0.04	1.16	7.08
9	76.51	1.21	0.05	1.23	6.89
12	76.37	1.21	0.05	1.24	6.98
18	77.12	1.14	0.05	1.36	6.59
24	75.47	1.14	0.04	1.56	6.68

[a] Results are presented as the average of 2 samples from each of 3 lots of Ester-C® Calcium Ascorbate.
[b] Samples were not tested for threonic acid at the 1-month timepoint.

Figure II.D-1 Ascorbic Acid Content of 3 Lots of Ester-C® Calcium Ascorbate Over a 24-Month Period



1.2 Accelerated Study

The Ester C Company also assessed the stability of Ester-C® under controlled accelerated storage conditions consisting of a temperature of 40°C and a relative humidity of 75%. Duplicate samples (A and B) from each of 3 lots (Lot Nos. 999-0605-001, 999-0605-002, 999-0605-003) at each time point were assessed for ascorbic acid, threonic acid, hydroxymethyl furanone, and free moisture content as well as for pH. An acceleration factor was obtained using the calculation-based acceleration model described by Meeker *et al.* (1998), and is dependent on 2 temperature levels (T_1 and T_2) and the activation energy (E_a) for the chemical in question. Using the acceleration model, where T_1 = 313.16 K (40°C) and T_2 = 298.16 K (25°C), and E_a for ascorbic acid = 83.144 kJ/mole, an acceleration factor of 5 was calculated. Thus, the constituent concentrations in samples stored under accelerated conditions for 365 days were predicted to be equivalent to those stored at 25°C for 1,800 days or 5 years. The study results showed that beginning at 36 months, the ascorbic acid content of several samples was reduced to below the specification range. The hydroxymethyl furanone content also began to decline beginning at 30 months in the majority of samples. In addition, the pH of half of the Ester-C® samples was increased to above the specification range beginning at 36 months, while the pH of the remaining samples was maintained within specification. In contrast, threonic acid and free moisture levels were maintained within specification throughout the course of the study. A package failure occurred at 18 months for lot No. 999-0605-003 sample B, evidenced by hard caking of the product and aberrant results. The results of this study are presented in Table II.D-2 and those for ascorbic acid also are presented in Figure II.D-2.

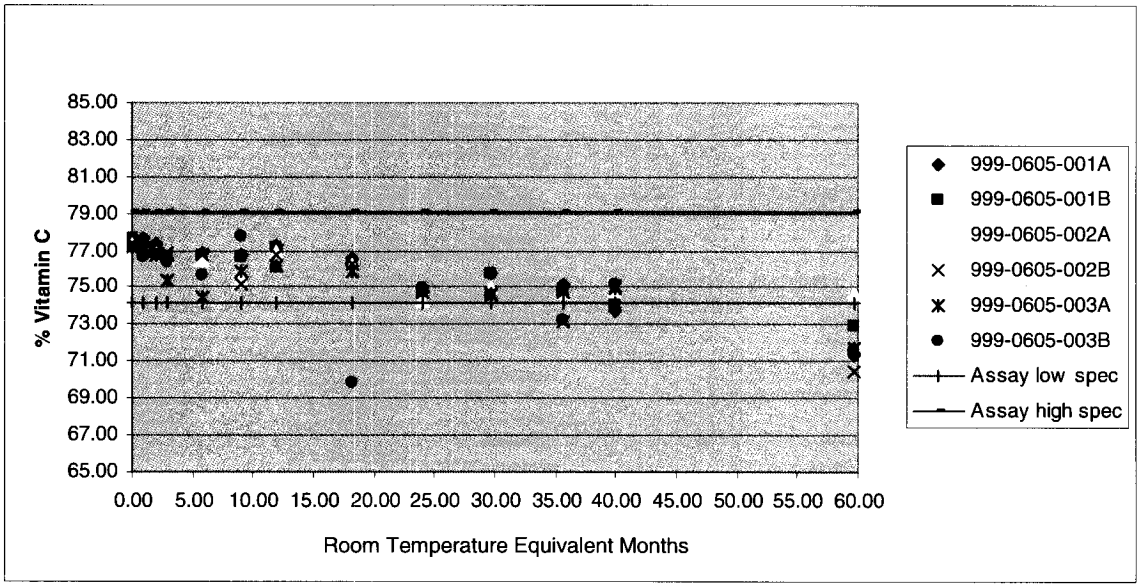
Based on the results of the accelerated study, a 24-month (2-year) shelf life was established, which is supported by the stability study conducted in real time at room temperature. Stability testing was conducted in accordance with current Good Manufacturing Practices (cGMP).

Table II.D-2 Stability of Ester-C® Calcium Ascorbate Over a 60-Month Period Under Accelerated Conditions

Storage Term (Projected accelerated months)	Content of Ester-C® Calcium Ascorbate Constituents[a]				
	Ascorbic acid (%)	Threonic acid[b] (%)	Hydroxymethyl furanone (%)	Free moisture (%)	pH
0.00	77.42	1.17	0.04	1.21	6.94
1	77.14	Not tested	0.04	1.10	6.94
2	76.98	Not tested	0.05	1.14	6.97
3	76.38	Not tested	0.05	1.13	7.02
6	76.14	1.09	0.05	1.21	7.04
9	76.27	1.15	0.05	1.22	7.06
12	76.73	1.16	0.05	1.30	7.05
18	75.18	1.25	0.04	1.60	6.87
24	74.71	1.21	0.04	1.36	7.16
30	75.04	1.27	0.03	1.32	7.25
36	74.26	1.17	0.03	1.52	7.31
40	74.62	1.20	0.02	1.39	7.22
60	72.13	1.26	0.01	0.99	6.95

[a] Results are presented as the average of 2 samples from each of 3 lots of Ester-C® Calcium Ascorbate.
[b] Samples were not tested for threonic acid at 1,2, and 3 months.

Figure II.D-2 Ascorbic Acid Content of 3 Lots of Ester-C® Calcium Ascorbate Over a 60-Month Period Under Accelerated Conditions



2. Stability Under the Intended Uses

2.1 Powdered Drink Mixes

A study was conducted to assess the stability of ascorbic acid from Ester-C® under the conditions of addition to a powdered mix drink. Ester-C® was blended into the powdered drink mix in an amount of 163 to 245 mg/g in order to provide 125 to 188 mg/g of ascorbic acid. The powdered mix was not reconstituted. The ascorbic acid content was determined at the start of the study (initial content) and at 6, 12, and 24 months by high performance liquid chromatography (HPLC). Beta carotene, vitamin B6, and niacin content also were assessed along with the physical appearance of the powder and microbiological parameters. As shown in Table II.D-3, the initial ascorbic acid content of the powdered mix following processing was 187 mg/g. The ascorbic acid content remained stable throughout the duration of the study, conforming to the established specifications for the powdered drink mix product. Thus, ascorbic acid from Ester-C® was demonstrated to be stable in a powdered drink mix for at least 24 months following addition by blending. All other parameters assessed also conformed to the established specifications throughout the duration of the 24-month study.

Table II.D-3 Stability of Ascorbic Acid from Ester-C® Calcium Ascorbate and Other Components in a Powdered Drink Mix

Parameter	Specification	Method of Analysis	Analyses Following Storage			
			0 (initial)	6 months	12 months	24 months
Ascorbic acid	125 to 188 mg/g	HPLC	187	160	162	182
Beta carotene	298 to 487 IU/g	HPLC	343	ND	357	406
Vitamin B6	2.50 to 3.75 mg/g	HPLC	3.23	3.02	3.05	3.02
Niacin	1.25 to 1.88 mg/g	HPLC	1.34	1.27	1.27	1.36
Appearance	Tan speckled powder with orange odor	Visual/olfactory	Conforms	Conforms	Conforms	Conforms
Total plate count	NMT 3,000 CFU/g	USP <2021>	Pass	ND	ND	Pass
Total yeast and mold count	NMT 300 CFU/g	USP <2021>	Pass	ND	ND	Pass
Staphylococcus aureus	Negative	USP <2021>	Pass	ND	ND	Pass
Escherichia coli	Negative	USP <2021>	Pass	ND	ND	Pass
Salmonella species	Negative	USP <2021>	Pass	ND	ND	Pass

CFU = colony forming units; HPLC = high performance liquid chromatography; ND = not determined; NMT = not more than; USP = United States Pharmacopeia

2.2 Beverages

The stability of ascorbic acid from Ester-C® following direct addition of the ingredient to beverages was assessed using orange juice as the representative beverage. Ester-C® was added to 2 different brands of orange juice (referred to as Brand A and Brand B, respectively) in

an amount of 72 mg of Ester-C® per 8 oz serving, which is equivalent to the Reference Amounts Customarily Consumed (RACC) per eating occasion for fruit juices as outlined by the FDA (21 CFR §101.12) (U.S. FDA, 2012a). According to their labels, the amount of vitamin C already present in each brand of orange juice was 78 mg per 8 oz, resulting in a total vitamin C content of 150 mg per 8 oz serving. The orange juice samples were stored for short-term periods of 1 to 35 days at 4°C. The vitamin C content of orange juice samples was then analyzed by iodometric titration daily over a period of 5 days and on the day of orange juice expiration (Day 35). A comparison of pH at purchase and at expiration of all samples also was conducted to assess any changes in orange juice acidity. Additionally, samples were observed for color change and precipitation of the Ester-C® ingredient.

The vitamin C content of both brands of orange juice following addition of Ester-C® remained stable for the duration of the storage period (up to 35 days) at 4°C and was comparable to or superior to the stability of vitamin C already present in the product (*i.e.*, orange juice with no added Ester-C®) (Table II.D-4). The addition of Ester-C® did not alter the acidity of either brand of orange juice as pH levels were maintained at approximately 3.90 for all samples. No change in color was observed in any of the samples over the 5-day period. All orange juice samples were only slightly darker at expiration (2008/03/25 for Brand A and 2008/03/27 for Brand B) compared to at purchase, and color changes were not perceptively different between the control and Ester-C®-supplemented samples. In addition, no precipitation of Ester-C® from the orange juice was observed for either brand during the 5-day period or at expiration. Moreover, upon a survey of 4 human participants, no clear preference in taste was detected between the control and Ester-C®-supplemented orange juice samples for both brands. Thus, Ester-C® did not negatively affect the overall taste quality of these brands of orange juice. The results of this study confirm the stability of Ester-C® in 2 brands of orange juice under the intended storage conditions.

Table II.D-4 Short-Term Stability of Ester-C® Calcium Ascorbate in Orange Juice

Storage Term (days)	Vitamin C Content (%)[a]			
	Orange Juice Brand A		Orange Juice Brand B	
	Without Ester-C®	With Ester-C®	Without Ester-C®	With Ester-C®
1	0.053	0.071	0.054	0.074
2	0.048	0.071	0.053	0.071
3	0.052	0.078	0.064	0.078
4	0.071	0.074	0.049	0.074
5	0.052	0.071	0.062	0.076
35	0.034	0.067	0.037	0.060

[a] Expressed as a percent of the beverage product.

In a second study, the stability of Ester-C® was compared to ascorbic acid in a fruit-flavored beverage system. Cranberry fruit juice concentrate was fortified with Ester-C® at 100%

(500 mg/240 mL) and 200% (750 mg/240 mL) overages, or an equivalent amount of ascorbic acid. Ester-C® or ascorbic acid was added at the start of the manufacturing process (*i.e.*, when other ingredients were added). The vitamin C content of each sample was analyzed in duplicate using reverse-phase HPLC with UV-diode array detection. The uncertainty of the vitamin C detection method was ±20%, based on a control sample. The data demonstrate that Ester-C® is as stable as ascorbic acid under the typical processing conditions applied to cranberry fruit juice concentrate (see Table II.D-5).

Table II.D-5 Stability of Ascorbic Acid From Ester-C® Calcium Ascorbate in Cranberry Juice Concentrate Following Processing (Ambient Conditions)

Sample	Analyses Following Processing	
	Vitamin C Content (mg/100 mL) [a]	% of Initial Added
Control	<1	n/a
Ester-C® (500 mg/240 mL)	215	43
Ester-C® (750 mg/240 mL)	319	42.5
Ascorbic acid (500 mg/240 mL)	204	40.8
Ascorbic acid (750 mg/240 mL)	293.5	39.1

n/a = not applicable
[a] Average value of duplicate samples.

Samples of the cranberry fruit juice concentrate were stored under ambient conditions (20°C±2), under artificial light, or chilled (4°C) in the dark for a period of 6 months. As shown in Table II.D-6, the results indicate that the stability of Ester-C® in cranberry fruit juice concentrate is comparable to that of ascorbic acid under the intended storage conditions.

Table II.D-6 Stability of Ester-C® Calcium Ascorbate Compared to Ascorbic Acid in Cranberry Juice Concentrate

Storage Term (months)	Vitamin C Content (%)[a]			
	100% Overage		200% Overage	
	Ester-C®	Ascorbic acid	Ester-C®	Ascorbic acid
Ambient (20°C±2), artificial light				
0	215	204	319	293
1	190.5	179.7	290.5	270.5
2	192	185	291.5	265.5
4	163	159.5	234.5	248.5
6	159	156.5	246	241
Chilled (4°C), dark				
1	199.3	187.1	297.1	274
2	211[b]	199[b]	316[b]	283.5[b]
4	197	181.5	291	272
6	204	194	312	293.5

[a] Average value of duplicate samples.
[b] Increase at 2-month time point was due to method uncertainty as opposed to an actual increase in vitamin C content.

2.3 Grain-Based Bars

The stability of ascorbic acid from Ester-C® in grain-based bars following addition under normal processing conditions for these types of food products was assessed using protein bars as the representative food. To this end, Ester-C® was added to 100 g of "Pure Protein Chocolate Peanut Butter" bar dough in amounts of 326, 408, and 490 mg, providing 250, 213, and 375 mg of ascorbic acid. These amounts represent overages of 100, 105, and 200% of a typical desired ascorbic acid content of 125 mg/100 g bar. A control bar sample, with no vitamin C or Ester-C® added, was included in the study. The bar dough samples were mixed and left to stand for 2 hours. Bars were then formed and coated with chocolate and the content of ascorbic acid was analyzed by HPLC. The test was conducted in duplicate and the average ascorbic acid contents summarized in Table II.D-7. The data demonstrate that Ester-C® is relatively stable under the typical processing conditions applied to grain-based bars, with a post-processing content of 70 to 73% of the initial amount of Ester-C® added. This in turn corresponds to a post-processing ascorbic acid content of 72 to 77% of the initial amount added as provided by Ester-C®. The data also demonstrate that an overage of 100% would support a final content of 125 mg ascorbic acid/100 g bar.

Table II.D-7 Stability of Ascorbic Acid From Ester-C® Calcium Ascorbate in a Protein Bar

Amount of Ester-C® Added (mg/100 g bar)	Corresponding Amount of Ascorbic Acid Added (mg/100 g bar)	Analyses Following Processing			
		Ester-C®		Ascorbic Acid	
		Content (mg/100 g bar)	Percent of Initial Added (%)	Content (mg/100 g bar)	Percent of Initial Added (%)
0 (control)	0	0	n/a	0	n/a
326	250	174	70	185	74
408	213	220	71	240	77
490	375	273	73	269	72

n/a = not applicable

Based on the above-described results, Ester-C® is expected to be stable under the intended conditions of use.

III Self-Limiting Levels of Use

Self-limiting use levels are not known

IV Detailed Summary of the Basis for Ester-C®'s GRAS Determination

The Ester C Company's GRAS determination that the intended nutritive food uses of Ester-C® as described in Table I.D-1, is based on generally available information supporting the safety of the constituent products of the ingredient: calcium ascorbate, calcium L-threonate, and hydroxymethyl furanone. The safety of dietary ascorbic acid, calcium ascorbate, threonic acid, hydroxymethyl furanone, and combinations thereof, have been evaluated by multiple authoritative and government regulatory bodies world-wide. Information provided by these opinions, and by published references cited therein was considered sufficient to determine that the proposed food uses of Ester-C® are GRAS based on scientific procedures. A summary of the data and information available within authoritative opinions and evaluations as it pertains to the safety of Ester-C, under the conditions of intended use in food, is discussed in section IV.B. Published studies detailing the metabolic fate, and toxicity of ascorbic acid, threonic acid and hydroxymethyl furanone are summarized in sections IV.C through IV.E.

The toxicity of Ester-C® has been evaluated in acute and repeat-dose studies using mature Sprague-Dawley rats. The mutagenicity of Ester-C® also has been evaluated *in vitro* using *Salmonella* and *Escherichia coli* tester strains. Studies evaluating the administration of Ester-C® to healthy human subjects also have been reported in the literature. The results of these studies are consistent with available information obtained from authoritative opinions and other generally available information within the literature, which demonstrate that consumption of ascorbic acid, calcium threonate and hydroxymethyl furanone from the intended conditions of

use of Ester-C® is generally recognized as safe. Safety information obtained from *in vitro* mutagenicity studies, data from rodent toxicity testing and published studies evaluating the consumption of Ester-C® by human subjects were therefore considered corroborative of safety.

Finally, the totality of data and information presented herein were reviewed by a Panel of Experts, qualified by scientific training and experience to evaluate the safety of ingredients as components of food, who similarly concluded that the intended uses of Ester-C® are GRAS based on scientific procedures [see Section IV.H].

IV.A Probable Consumption Estimates

1. Estimated Consumption of Ascorbic Acid, Calcium, Threonic Acid, and Hydroxymethyl Furanone from Natural Occurrences in Food

Calcium ascorbate is ascorbic acid buffered by calcium (*i.e.*, a calcium salt of ascorbic acid), and thus, is essentially ascorbic acid and calcium together in salt form. Both ascorbic acid and calcium have well-established and long histories of human consumption as both occur naturally in many foods. Ascorbic acid is an essential vitamin that occurs predominantly in fruits and vegetables, and calcium is an essential mineral that occurs predominantly in dairy products. The Recommended Dietary Allowance (RDA) for vitamin C in the U.S. is 90 mg/day for adult men and 75 mg/day for adult women as established by the Food and Nutrition Board of the Institute of Medicine (IOM, 2000). Using the NHANES for the years 1988 to 1994, the Food and Nutrition Board of the IOM estimated that the mean and 90th percentile background dietary intakes of vitamin C by the U.S. population ranged from 90 to 140 mg/person/day and 120 to 210 mg/person/day, respectively, depending on the population group (IOM, 2000, Appendix C). For the total population, the mean and 90th percentile background dietary intakes of vitamin C were reported to be 106 mg/person/day and 150 mg/person/day, respectively (IOM, 2000, Appendix C). When supplemental use was included in the calculation, the mean and 90th percentile intakes of vitamin C were estimated to range from 100 to 200 mg/person/day and 140 to 420 mg/person/day, respectively, depending on the population group. For the total population, the mean and 90th percentile intakes of vitamin C from the diet and supplemental use were reported to be 167 mg/person/day and 246 mg/person/day, respectively. For calcium, the IOM set an Adequate Intake (AI) of 1,000 mg/day for adult men and women. Based on data from the 1994 Continuing Survey of Food Intakes by Individuals (CSFII), a national survey on 2-day food and nutrient intakes, mean and 90th percentile intakes of calcium were estimated to range from 460 to 1,170 mg/person/day and 670 to 2,040 mg/person/day, respectively, depending on the population group (IOM, 1997, Appendix D). For the total population, the mean and 90th percentile intakes of calcium were reported to be 797 mg/person/day and 1,241 mg/person/day, respectively. In addition, calcium ascorbate itself appears on the FDA's list of food ingredients that are GRAS for use in food for human consumption. It is inherent in

this GRAS status then that calcium ascorbate has a long history of use in food as a food ingredient.

L-Threonic acid is a known oxidative degradation product of ascorbic acid (Tatum *et al.*, 1969; Deutsch, 1998; Knafo *et al.*, 2005; Debolt *et al.*, 2007), and thus, occurs naturally in foods that contain ascorbic acid, which establishes a history of human consumption for this compound. Threonic acid also has been detected in bread (Thewlis, 1971) as well as in drinking water (Jansson *et al.*, 2004).

Hydroxymethyl furanone has a well-established and long history of human consumption as it occurs naturally in many cooked foods as a result of the Maillard reaction that takes place between pentose sugars and amino acids during cooking (Slaughter, 1999). Such foods include, but are not limited to, beef broth, roasted sesame seeds, bread crust, soy sauce, malt, beer, popcorn, and cooked clam (Slaughter, 1999), in which hydroxymethyl furanone is an important natural component of food flavor and aroma. In addition, hydroxymethyl furanone is present in the aroma of blackberries (Klesk and Qian, 2003) and occurs naturally in guava and raspberries (Burdock, 2009), the latter of which has a hydroxylmethyl furanone content of 0.1 ppm as reported by FEMA. Tomatoes also have a content of naturally occurring hydroxymethyl furanone with a concentration of 10 mg/kg tomato (1 ppm) (Buttery *et al.*, 1994). Furthermore, as hydroxymethyl furanone is GRAS by FEMA, it can be assumed that there is a history of use of this ingredient as a flavoring agent in food products. FEMA reports the Possible Average Daily Intake (PADI) of hydroxymethyl furanone by the U.S. population as 0.84 mg from a variety of food sources, including condiment relish, fats and oils, fish products, gelatin pudding, gravies, meat products, nut products, poultry, seasonings and flavors, snack foods, soups, and sweet sauces. Additionally, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has reported daily intakes of hydroxymethyl furanone as a flavoring agent of 56 and 0.07 µg/person/day, or 0.9 and 0.001 µg/kg body weight/day, for the European and U.S. populations, respectively, based on annual production volume (JECFA, 2006b).

2. Estimated Consumption of Ester-C® from Intended Food Uses

Estimates for the intake of Ester-C® were based on the intended food uses and use levels (Table I.D-1) in conjunction with food consumption data included in the NCHS 2003-2004 NHANES (CDC, 2006; USDA, 2009). NHANES are conducted as a continuous, annual survey, and are released in 2-year cycles. Each year about 7,000 people from 15 different locations across the U.S. are interviewed, and approximately 5,000 people complete the health examination component of the survey. Any combination of consecutive years of data collection is a nationally-representative sample of the U.S. population. The surveys provide the most appropriate data for evaluating food use and food consumption patterns in the United States, containing 2 years of data on individuals selected *via* a stratified multistage probability sample of the civilian non-institutionalized population of the U.S. It is well established that the length of a

dietary survey affects the estimated consumption of foods by individual users and that short-term surveys, such as the typical 1-day dietary survey, overestimate consumption over longer time periods (Anderson, 1988). Two 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) are available from the NHANES 2003-2004 survey, and thus, these data were used to generate estimates for the current intake analysis.

NHANES 2003-2004 survey data were collected from individuals and households *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout all 4 seasons of the year. Day 1 data were collected in person, and Day 2 data were collected by telephone in the following 3 to 10 days, on different days of the week, to achieve the desired degree of statistical independence. The data were collected by first selecting Primary Sampling Units (PSUs), which were counties throughout the U.S. Small counties were combined to attain a minimum population size. These PSUs were segmented and households were chosen within each segment. One or more participants within each household were interviewed. Fifteen PSUs are visited each year. For NHANES 2003-2004, 12,761 individuals were selected for the sample, and 10,122 were interviewed (79.3%).

In addition to information on the types and quantities of foods being consumed, NHANES 2003-2004 involved the collection of socioeconomic, physiological, and demographic information from individual participants in the survey, such as sex, age, height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population. Sample weights were incorporated with NHANES 2003-2004 to compensate for the potential under-representation of intakes from specific population groups as a result of sample variability due to survey design, differential non-response rates, or other factors, such as deficiencies in the sampling frame (CDC, 2006; USDA, 2009).

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of Ester-C® by the U.S. population under the conditions of intended use. Estimates for the daily intake of Ester-C® represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2003-2004 data; these average amounts comprised the distribution from which mean and percentile intake estimates were produced. Mean and percentile estimates were generated by incorporating survey weights in order to provide representative intakes for the entire U.S. population. All-person intake refers to the estimated intake of Ester-C® averaged over all individuals surveyed, regardless of whether they consumed food products containing Ester-C®, and therefore, includes "zero" consumers (those who reported no intake of food products containing Ester-C® during the 2 survey days). All-user intake refers to the estimated intake of Ester-C® only by those individuals consuming food products containing Ester-C®, hence the "all-user" designation. Individuals were considered users if they consumed 1 or more food products containing Ester-C® on either Day 1 or Day 2 of the survey.

Calculations for the mean and 90th percentile all-person and all-user intakes, and percent of the population consuming were performed for each of the individual intended food uses of Ester-C®. Similar calculations were used to determine the estimated total intake of Ester-C® from all intended food uses combined. In both cases, the per person and per kilogram body weight intakes were reported for the following population groups:

infants, ages 0 to 2;
children, ages 3 to 11;
female teenagers, ages 12 to 19;
male teenagers, ages 12 to 19;
female adults, ages 20 and up;
male adults, ages 20 and up; and,
total population (all population and gender groups combined).

Estimates for the total daily intake of Ester-C® from all intended food uses are provided in Tables IV.A-1 and IV.A-2 on a per person and per kilogram body weight basis, respectively. Although, the Ester C Company intends to use Ester-C® in medical foods at use levels providing up to 500 mg of vitamin C per serving, the estimated intakes of Ester-C® did not take into consideration this specific food use.

Approximately 71.1% of the total U.S. population was identified as potential consumers of Ester-C® from the intended food uses (5,876 actual users identified). Children were determined to be the greatest percentage of users of the population groups at 88.1% and male adults were determined to be the lowest percentage of users of any population group at 63.4%. Large user percentages within a population group typically lead to similar results for the all-person and all-user consumption estimates, and in such instances, only the all-user intake results are generally discussed in detail. Although, the user percentages for Ester-C® for each population group did not approach 100%, the percentages remained sufficiently large, for the most part, to result in all-person and all-user estimated intakes of Ester-C® that were not altogether dissimilar. Therefore, only the all-user intake results will be discussed in detail so as to provide a conservative estimate of the estimated intakes of Ester-C® under the conditions of intended use.

On an all-user basis, the mean intake of Ester-C® by the total U.S. population from all intended food uses was estimated to be 373 mg/person/day or 8 mg/kg body weight/day (Tables IV.A-1 and IV.A-2). The heavy consumer (90th percentile) all-user intake of Ester-C® by the total U.S. population from all intended food uses was estimated to be 810 mg/person/day or 18 mg/kg body weight/day.

Table IV.A-1 Summary of the Estimated Daily Intake of Ester-C® from All Intended Food uses in the U.S. by Population Group (2003-2004 NHANES Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (mg)	90th Percentile (mg)	Mean (mg)	90th Percentile (mg)
Infants	0 to 2	63.9	594	279	763	395	839
Children	3 to 11	88.1	1,134	327	763	388	839
Female Teenagers	12 to 19	75.2	746	269	684	375	762
Male Teenagers	12 to 19	75.4	753	351	938	481	1,053
Female Adults	20 and Up	67.0	1,426	208	579	328	708
Male Adults	20 and Up	63.4	1,223	234	619	391	882
Total Population	All Ages	71.1	5,876	247	639	373	810

Table IV.A-2 Summary of the Estimated Daily per Kilogram Body Weight Intake of Ester-C® from All Intended Food uses in the U.S. by Population Group (2003-2004 NHANES Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (mg/kg)	90th Percentile (mg/kg)	Mean (mg/kg)	90th Percentile (mg/kg)
Infants	0 to 2	63.9	594	23	61	32	69
Children	3 to 11	88.1	1,134	14	33	16	35
Female Teenagers	12 to 19	75.2	746	5	12	6	14
Male Teenagers	12 to 19	75.4	753	5	14	7	17
Female Adults	20 and Up	67.0	1,426	3	8	5	10
Male Adults	20 and Up	63.4	1,223	3	7	5	10
Total Population	All Ages	71.1	5,876	5	13	8	18

On an individual population basis, the greatest mean all-user intakes of Ester-C® on an absolute basis were estimated to be in male teenagers at 481 mg/person/day (Table IV.A-1). Female adults were estimated to have the lowest mean all-user intakes of Ester-C® on an absolute basis, with a value of 328 mg/person/day. On a per kilogram body weight basis, the mean all-user intakes of Ester-C® were estimated to be highest in infants, with intakes of 32 mg/kg body weight/day (Table IV.A-2). The lowest all-user mean intake on a body weight basis was estimated to be in female and male adults, with a value of 5 mg/kg body weight/day.

When heavy consumers (90th percentile) were assessed, all-user intakes of Ester-C® from all intended food uses also were estimated to be greatest in male teenagers, with a value of 1,053 mg/person/day (Table IV.A-1). The estimated lowest 90th percentile all-user intakes were in female adults, with a value of 708 mg/person/day on an absolute basis. On a body weight basis, infants were estimated to have the greatest all-user 90th percentile intakes of Ester-C®,

with a value of 69 mg/kg body weight/day (Table IV.A-2). The estimated lowest all-user 90th percentile intakes of Ester-C® on a body weight basis were observed in both female and male adults at 10 mg/kg body weight/day.

3. Estimated Consumption of Vitamin C and Other Constituents from Intended Food Uses of Ester-C® Calcium Ascorbate

Estimates for the daily intake of vitamin C from all intended food uses of Ester-C® were calculated for the U.S. population based on the estimated daily intakes of Ester-C® presented in Section IV.A.2. The estimated intakes for vitamin C were calculated using the ascorbic acid content of Ester-C®, which is 76.60%. These data are summarized in Tables IV.A-3 and IV.A-4 on a per person and per kilogram body weight basis, respectively. For the total U.S. population, the estimated mean all-user intake of vitamin C from all intended food uses of Ester-C® was calculated to be 286 mg/person/day or 6 mg/kg body weight/day (Tables IV.A-3 and IV.A-4). The 90th percentile all-user intake of vitamin C by the total population was estimated to be 620 mg/person/day or 14 mg/kg body weight/day.

On an individual population basis, the greatest mean all-user intakes of vitamin C on an absolute basis were estimated to occur in male teenagers at 369 mg/person/day (Table IV.A-3). Female adults had the lowest estimated mean all-user intake of vitamin C on an absolute basis, with a value of 252 mg/person/day. On a per kilogram body weight basis, mean all-user intakes of vitamin C were estimated to be highest in infants, with an intake of 24 mg/kg body weight/day (Table IV.A-4). The lowest all-user mean intake of vitamin C on a body weight basis was 4 mg/kg body weight/day, as estimated to occur in both female and male adults.

When 90th percentile consumers were assessed, all-user intakes of vitamin C also were estimated to be greatest in male teenagers at 806 mg/person/day (Table IV.A-3). The lowest 90th percentile all-user intake of vitamin C on an absolute basis was estimated to be in female adults, with a value of 542 mg/person/day. On a body weight basis, infants were estimated to have the greatest all-user 90th percentile intakes of vitamin C, with a value of 53 mg/kg body weight/day (Table IV.A-4). The lowest all-user 90th percentile intake of vitamin C on a body weight basis was 7 mg/kg body weight/day, as estimated to occur in both female and male adults.

Table IV.A-3 Summary of the Estimated Daily Intake of Vitamin C[a] from All Intended Food Uses of Ester-C® in the U.S. by Population Group (2003-2004 NHANES Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (mg)	90th Percentile (mg)	Mean (mg)	90th Percentile (mg)
Infants	0 to 2	63.9	594	213	584	302	642
Children	3 to 11	88.1	1,134	251	585	297	643
Female Teenagers	12 to 19	75.2	746	206	524	287	583
Male Teenagers	12 to 19	75.4	753	269	718	369	806
Female Adults	20 and Up	67.0	1,426	159	444	252	542
Male Adults	20 and Up	63.4	1,223	180	474	300	676
Total Population	All Ages	71.1	5,876	189	489	286	620

[a] All-person and all-user consumption data for vitamin C are calculated from the Ester-C® consumption data based on its composition (76.6% of Ester-C®).

Table IV.A-4 Summary of the Estimated Daily per Kilogram Body Weight Intake of Vitamin C[a] from All Intended Food Uses of Ester-C® in the U.S. by Population Group (2003-2004 NHANES Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (mg/kg)	90th Percentile (mg/kg)	Mean (mg/kg)	90th Percentile (mg/kg)
Infants	0 to 2	63.9	594	17	46	24	53
Children	3 to 11	88.1	1,134	10	26	12	27
Female Teenagers	12 to 19	75.2	746	4	9	5	10
Male Teenagers	12 to 19	75.4	753	4	10	6	13
Female Adults	20 and Up	67.0	1,426	2	6	4	7
Male Adults	20 and Up	63.4	1,223	2	6	4	7
Total Population	All Ages	71.1	5,876	4	10	6	14

[a] All-person and all-user consumption data for vitamin C are calculated from the Ester-C® consumption data based on its composition (76.6% of Ester-C®).

Estimates for the daily intake of calcium, threonic acid, and hydroxymethyl furanone from all intended food uses of Ester-C® also were calculated for the U.S. population based on the estimated of daily intakes of Ester-C®. These estimated intakes are summarized in Tables IV.A-5 and IV.A-6 on an absolute and on a body weight basis, respectively.

Table IV.A-5 Summary of the Estimated Daily Intake of Calcium, Threonic Acid, and Hydroxymethyl Furanone[a] from All Intended Food Uses of Ester-C® in the U.S. by Population Group (2003-2004 NHANES Data)

Population Group	Age Group (Years)	Calcium				Threonic Acid				Hyroxymethyl Furanone			
		All-Person Consumption		All-User Consumption		All-Person Consumption		All-User Consumption		All-Person Consumption		All-User Consumption	
		Mean (mg)	90th Percentile (mg)	Mean (mg)	90th Percentile (mg)	Mean (mg)	90th Percentile (mg)	Mean (mg)	90th Percentile (mg)	Mean (µg)	90th Percentile (µg)	Mean (µg)	90th Percentile (µg)
Infants	0 to 2	26	70	36	77	3	9	5	10	125	343	178	377
Children	3 to 11	30	70	36	77	4	9	4	10	147	344	175	377
Female Teenagers	12 to 19	25	63	34	70	3	8	4	9	121	308	169	343
Male Teenagers	12 to 19	32	86	44	97	4	11	6	12	158	422	217	474
Female Adults	20 and Up	19	53	30	65	2	7	4	8	94	261	148	318
Male Adults	20 and Up	22	57	36	81	3	7	4	10	105	278	176	397
Total Population	All Ages	23	59	34	74	3	7	4	9	111	287	168	364

[a] All-person and all-user consumption data for calcium, threonic acid, and hydroxymethyl furanone are calculated from the Ester-C® consumption data based on its composition (9.18%, 1.15%, and 0.045% of Ester-C®, respectively).

Table IV.A-6 Summary of the Estimated Daily per Kilogram Body Weight Intake of Calcium, Threonic Acid, and Hydroxymethyl Furanone[a] from All Intended Food uses of Ester-C® in the U.S. by Population Group (2003-2004 NHANES Data)

Population Group	Age Group (Years)	Calcium				Threonic Acid				Hyroxymethyl Furanone			
		All-Person Consumption		All-User Consumption		All-Person Consumption		All-User Consumption		All-Person Consumption		All-User Consumption	
		Mean (mg/kg)	90th Percentile (mg/kg)	Mean (mg/kg)	90th Percentile (mg/kg)	Mean (µg/kg)	90th Percentile (µg/kg)	Mean (µg/kg)	90th Percentile (µg/kg)	Mean (µg/kg)	90th Percentile (µg/kg)	Mean (µg/kg)	90th Percentile (µg/kg)
Infants	0 to 2	2.07	5.56	2.94	6.37	260	697	368	798	10	27	14	31
Children	3 to 11	1.25	3.06	1.49	3.21	157	383	186	402	6	15	7	16
Female Teenagers	12 to 19	0.42	1.09	0.59	1.25	53	136	74	157	2	5	3	6
Male Teenagers	12 to 19	0.49	1.25	0.68	1.53	62	157	85	192	2	6	3	8
Female Adults	20 and Up	0.27	0.70	0.42	0.87	34	88	53	109	1	3	2	4
Male Adults	20 and Up	0.25	0.68	0.42	0.89	32	85	53	112	1	3	2	4
Total Population	All Ages	0.49	1.24	0.73	1.66	61	155	92	208	2	6	4	8

[a] All-person and all-user consumption data for calcium, threonic acid, and hydroxymethyl furanone are calculated from the Ester-C® consumption data based on its composition (9.18%, 1.15%, and 0.045% of Ester-C®, respectively).

4. Summary

In summary, consumption data and information pertaining to the individual intended food uses were used to estimate the all-person and all-user intakes of Ester-C® for specific demographic groups and for the total U.S. population. This type of intake methodology is generally considered to be 'worst case' as a result of several conservative assumptions made in the consumption estimates. For example, it is often assumed that all food products within a food category contain the ingredient at the maximum specified level of use. Thus, the estimated intakes of Ester-C® are over-estimates of anticipated actual consumption. In addition, it is well established that the length of a dietary survey affects the estimated consumption of individual users. Short-term surveys, such as the typical 2- or 3-day dietary surveys, overestimate the consumption of food products that are consumed relatively infrequently. Therefore, it is anticipated that the actual intake of Ester-C® from the intended conditions of use will be less than estimated.

The per person mean and 90th percentile intakes (all-user basis) of Ester-C® and of the individual constituents of Ester-C® by the total U.S. population from all proposed food-uses is summarized below in Table IV.A-7. The background dietary intakes of the constituents also are provided in Table IV.A-7 for comparative purposes.

Table IV.A-7 Summary of the Estimated Daily Intake of Ester-C® and of the Individual Constituent from All Intended Food Categories in the United States by the Total Population on a All-User Basis (2003-2004 NHANES Data)

Ester-C® and its individual constituents	Background Dietary Intake by Total U.S. Population[a]		All-Users Consumption of Ester-C® by Total U.S. Population	
	Mean (mg)	90th Percentile (mg)	Mean (mg)	90th Percentile (mg)
Ester-C®	N/A	N/A	373	810
Vitamin C (ascorbic acid)	106 (90 to 140)	150 (120 to 210)	286	620
Calcium	797 (460 to 1,170)	1,241 (670 to 2,040)	34	74
Threonic acid	N/A	N/A	4	9
Hydroxymethyl furanone	0.00007[b] and 0.84[c]	N/A	0.168	0.364

N/A = not applicable
[a] Numbers in parentheses represent the range of intakes across all population groups.
[b] As reported by JECFA (2006b).
[c] As reported by FEMA.

As mentioned in Section IV.A.1, the mean and 90th percentile background dietary intakes of vitamin C by the total U.S. population were estimated by the IOM to be 106 mg/person/day and 150 mg/person/day, respectively (IOM, 2000). When supplemental use also was considered, the total mean and 90th percentile intakes of vitamin C were reported to be 167 mg/person/day

and 246 mg/person/day, respectively. In contrast, the total population all-user mean and 90th percentile daily intakes of vitamin C (286 and 620 mg/person/day, respectively) from all food uses of Ester-C® were estimated to be greater than the background daily intakes of vitamin C reported by the IOM. The estimated 90th percentile all-user consumption for vitamin C from all food uses of Ester-C® was greatest in the male teenager population group, with an intake of 806 mg/person/day. This estimated intake also is greater than the reported 90th percentile background vitamin C intake from dietary sources for male teenagers in the U.S., which is 199 mg/person/day (IOM, 2000). On a body weight basis, the highest 90th percentile all-user intake of vitamin C from all food uses of Ester-C® was estimated to occur in infants at 642 mg/person/day, which is greater than the 90th percentile background daily vitamin C intake of 120 to 147 mg/person/day reported for infants in the U.S. (IOM, 2000).

In addition, the estimated intakes of vitamin C are above the RDA of 90 mg/day for adult men and 75 mg/day for adult women established by the IOM (2000). Therefore, background dietary intake alone appears to be sufficient in achieving the RDA. Such background dietary intakes of vitamin C, however, include food and beverage sources to which vitamin C is added. As Ester-C® is intended, in part, to replace existing vitamin C fortificants in many foods and beverages, the intended use of Ester-C® will, in this respect, aid in achieving the RDA for vitamin C. Based on the intakes analysis, the intended use of Ester-C® may increase the total daily intakes of vitamin C as it is also intended for use in foods to which vitamin C is not currently added. Although the anticipated vitamin C intake from the intended conditions of use of Ester-C® are above current background intake levels, it is expected not to be of concern to human health considering that these intakes of vitamin C from Ester-C® are cumulative of smaller intakes taken with food throughout the day as opposed to a bolus intake, and remain below the Tolerable Upper Intake Level (UL) of 2,000 mg/person/day set by the IOM (2000). The safety of Ester-C® and vitamin C, as well as the other constituents, is further discussed in Sections IV.B through IV.G. Additionally, due to the nature of the method of intake calculation, these estimates likely overestimate the consumption of all population groups considering that all manufacturers are unlikely to use the maximum regulatory limit in all permitted food types and the short-term food consumption databases were used to estimate long-term consumption.

For calcium, the IOM reported that mean and 90th percentile intakes of calcium were estimated to be 797 mg/person/day and 1,241 mg/person/day, respectively, for the total U.S. population (see Section IV.A.1) (IOM, 1997). The estimated mean and 90th percentile intakes of calcium from all food uses of Ester-C® for the total population fall well below these values at 34 and 74 mg/person/day, respectively. In addition, the IOM set an AI of 1,000 mg/person/day for adult men and women and a UL of 2,500 mg/person/day for adult men and women between the ages of 19 and 50 years (IOM, 2011). Therefore, the the anticipated calcium intake from the intended conditions of use of Ester-C® is expected to have no significant effect on achieving the AI, and also is expected not to be of concern to human health.

Threonic acid and hydroxymethyl furanone are minor constituents of Ester-C® with estimated total population all-user mean intakes of 4 and 0.168 mg/person/day, respectively, from all food uses of Ester-C®. The estimated intakes at the 90th percentile for the total population were 9 and 0.364 mg/person/day for threonic acid and hydroxymethyl furanone, respectively. The background dietary intake of calcium threonate or threonic acid was not identified in the literature. For hydroxymethyl furanone, JECFA has reported daily intakes of hydroxymethyl furanone of 0.056 and 0.00007 mg/person/day for the European and U.S. populations, respectively (JECFA, 2006b) and FEMA reported a PADI of 0.84 mg by the U.S. population (see Section IV.A.1). Therefore, the total population estimated intakes of hydroxymethyl furanone from all food uses of Ester-C® are greater than those reported by JECFA, but fall within the intake reported by FEMA. Given that threonic acid and hydroxymethyl furanone occur in Ester-C® at minimal levels, and considering the available safety data as discussed in Sections IV.B through IV.G, these constituents are expected not to be of concern to human health from the intended conditions of use of Ester-C®.

IV.B Safety Evaluations Conducted by Scientific Committees

1. Ascorbic Acid and Calcium Ascorbate

The safety of ascorbic acid and/or calcium ascorbate has been reviewed by several recognized scientific committees, including JECFA, the Food and Nutrition Board of the IOM, the European Food Safety Agency (EFSA), and the United Kingdom's Expert Group on Vitamins and Minerals (EVM). The JECFA evaluated the safety of ascorbic acid and its calcium, potassium, and sodium salts for use as food additives and as vitamin C supplements in the usually accepted levels of intakes for nutritional purposes (JECFA, 1981). The Committee commented that animal studies demonstrated that ascorbic acid is not toxic after single or repeated administration of doses up to 2,500 mg/kg body weight/day, and that in humans, daily doses of 100 to 6,000 mg have been taken over short periods of time with no adverse effects. The Committee also noted that although oxalic acid is a urinary metabolite of ascorbic acid, the risk of crystalluria and the formation of calcium oxalate stones due to calcium ascorbate consumption are unlikely given the minor intake of calcium from this source compared to the total dietary intakes of calcium. Based on their evaluation, JECFA allotted an acceptable daily intake (ADI) of "not specified[1]" for ascorbic acid and its calcium, potassium, and sodium salts.

The IOM reviewed the human data on vitamin C, which indicated that adverse effects due to intakes of ascorbic acid supplements are limited to osmotic diarrhea and gastrointestinal disturbances, which are self-limiting (IOM, 2000). Specifically, upon human ingestion of large bolus doses of supplemental vitamin C, in the order of 3,000 mg or greater, gastrointestinal

[1] A term applicable to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological, and other), the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food does not, in the opinion of JECFA, represent a hazard to health (WHO, 1987).

adverse effects were reported in both case reports and studies. Such gastrointestinal disturbances include diarrhea, nausea, and abdominal cramps and are attributed to the osmotic effect of unabsorbed ascorbic acid passing through the gastrointestinal tract. Accordingly, the IOM selected a lowest-observed-adverse-effect level (LOAEL) of 3,000 mg/person/day for healthy adults based on reported symptoms of flatulent distension, transient colic, and diarrhea following the consumption of ascorbic acid in increasing increments of 1 g/day in successive weeks to an upper dose of 4 g/day by normal healthy volunteers (number not reported) (Cameron and Campbell, 1974). The occurrence of mild diarrhea in one of 3 subjects following the consumption of 4 g ascorbic acid/day (Stein *et al.*, 1976) and diarrhea in 2 of 15 subjects ingesting 10 g ascorbic acid/day (Wandzilak *et al.*, 1994), as well as case reports (Hoffer, 1971; Hoyt, 1980) were cited by the IOM as supporting evidence to suggest that osmotic diarrhea is likely to occur following the consumption of greater than 3 g ascorbic acid/day. Based on the available data, the IOM concluded that there was little doubt surrounding the level of intake of ascorbic acid that may cause diarrhea and selected an uncertainty factor of 1.5 for the derivation of a UL from the LOAEL. Thus, the UL for healthy adults aged 19 years and older, including pregnant or lactating women, was determined to be 2,000 mg/person/day. The ULs for children and adolescents were extrapolated on a body weight basis from the UL for adults as the results of the few studies available in these population groups indicated that on a body weight basis, the adverse effects were similar to those observed in adults. Thus, the ULs were determined to be 400, 650, and 1,200 mg/person/day for children aged 1 to 3 years, 4 to 8 years, and 9 to 13 years, respectively; and 1,800 mg/person/day for adolescents and pregnant or lactating female adolescents. The IOM was not able to establish a UL for infants due to insufficient data on the adverse effects of vitamin C in this age group. The ULs for vitamin C are applicable to intake from both food and supplements. To reiterate, the LOAEL from which the UL for adults was derived was based on gastrointestinal adverse effects observed following large bolus doses of supplemental vitamin C and not following smaller vitamin C intakes from food ingested throughout the day. Importantly, the IOM noted that the effects on which the UL was based are generally not serious and are self-limiting in that reducing supplemental vitamin C intakes will eliminate such effects. See Table IV.B-1 below for a summary of the ULs derived by the IOM. With respect to the other suspected adverse effects [*i.e.*, increased oxalate excretion and kidney stone formation, increased uric acid excretion, pro-oxidant effects, systemic conditioning ('rebound scurvy'), increased iron absorption leading to iron overload, reduced vitamin B_{12} and copper status, increased oxygen demand, and erosion of dental enamel], the IOM concluded that the data did not provide sufficient evidence or support for a causal relationship between their occurrence and ascorbic acid intake by healthy individuals.

Table IV.B-1 Tolerable Upper Intake Levels (UL) and the Estimated Daily Intake of Vitamin C from All Intended Food Uses of Ester-C® in the U.S. by Population Group

Population Group	Age Group (Years)	UL (mg/day)	All-User Consumption	
			Mean (mg)	90th Percentile (mg)
Children	1 to 2	400	302	642
	3	400	297	643
	4 to 8	650		
	9 to 11	1,200		
Female Teenagers	12 to 13	1,200	287	583
	14 to 18	1,800		
	19	2,000		
Male Teenagers	12 to 13	1,200	369	806
	14 to 18	1,800		
	19	2,000		
Female Adults	20 and Up	2,000	252	542
Male Adults	20 and Up	2,000	300	676
Total Population	All Ages	Not applicable	286	620

As shown in Table IV.A-3, the anticipated 90th percentile vitamin C intake from the intended conditions of use of Ester-C® are below the UL set by the IOM for each population group, except for young children ages 1 to 3 and children ages 4 to 8. In these latter 2 groups, although the estimated mean intakes of vitamin C from Ester-C® remain below the respective ULs of 400 and 650 mg/person/day, respectively, the estimated 90th percentile intakes of vitamin C of approximately 640 mg/person/day meet or exceed these ULs. As mentioned, the ULs for children were extrapolated from that established for adults based on body weight differences. This methodology was undertaken since the data on adverse effects in children were consistent with those in adults following bolus doses of supplemental vitamin C (Ludvigsson *et al.*, 1977). Findings from a recent randomized placebo controlled study in children and adolescents ages 2 through 16 with Charcot-Marie tooth disease administered vitamin C twice daily resulting in intakes equivalent to the subject's age specific UL for a period of 12 months, further demonstrate that twice daily consumption of bolus intakes of vitamin C by this population group does not result in adverse gastrointestinal effects or incidences of osmotic diarrhea (Burns *et al.*, 2009).

In contrast to the exposure occurring from supplement uses of vitamin C evaluated in clinical investigations, the estimated daily intakes of vitamin C from the intended conditions of use of Ester-C® in food and/or drink products will occur on a cumulative basis throughout the course of a day. Therefore, acute consumption of vitamin C from Ester-C® containing products by young children at quantities approaching or exceeding the UL for vitamin C is not expected to occur. For example, between 43.6 to 64.2% of the total estimated consumption of vitamin C among

consumers of Ester-C® containing foods between the ages of 2 through 12 is accounted for by consumption of processed fruits and fruit juices that may potentially contain Ester-C. Based on 2003-2004 NHANES data, the average intake of all fruit juices and fruit-flavored drinks and ades on a per consumption basis was 180 mL by infants aged 0 to 6 months, 144 mL by infants aged 7 to 12 months, 190 mL by children aged 1 to 3 years, and 223 mL by children aged 4 to 8 years. Based on this information, the average level of vitamin C intake from Ester-C-containing beverages by infants and children is estimated to be less than 200 mg per serving. The Ester C Company also notes that the 90th percentile exposure estimates are representative of cumulative exposures obtained using a heterogeneous group of heavy consumers of potential Ester-C® containing products and do not reflect a pattern of consumption by a given individual who is unlikely to be a heavy consumer of all foods across all food categories. Among heavy users, long-term habitual consumption patterns typically revert to the mean. Conservative all-user mean intakes of ascorbic acid among infants and children were estimated to be 302 and 297 mg/person/day respectively. Thus, the Ester C Company has concluded that acute or habitual intake patterns among young children that may consume Ester-C® containing products at the maximum use level would not exceed the established UL for vitamin C in these population groups.

Contrary to the IOM, the EFSA's Scientific Panel on Dietetic Products, Nutrition, and Allergies determined that there were insufficient data to establish a UL for vitamin C from ascorbic acid and its calcium, potassium, and sodium salts as well as from L-ascorbyl-6-palmitate (EFSA, 2004). The scientific panel concluded that ascorbic acid possesses low acute toxicity in animals and humans, and that, overall, acute gastrointestinal intolerances, such as diarrhea, abdominal distention, flatulence, and transient colic, were the most clearly defined adverse effects of high ascorbic acid intakes in humans. The panel noted, however, that limited data were available on the dose-response relationship for these effects and that very few controlled studies have been conducted to investigate the adverse effects of high-dose ascorbic acid intake. The panel further determined that the safety of long-term use of high-dose vitamin C supplements has not been evaluated. Following their review, the panel concluded that supplemental daily doses of vitamin C up to approximately 1,000 mg/person in addition to vitamin C intake from the diet was not associated with adverse gastrointestinal effects, but that such effects may occur at intakes of 3,000 to 4,000 mg/person/day and greater.

The EVM similarly concluded that insufficient data existed to set a Safe Upper Level for vitamin C. The EVM, however, did agree with the LOAEL of 3,000 mg/person/day proposed by the IOM, and concluded that a supplemental dose of 1,000 mg/person/day, or 17 mg/kg body weight/day, would not be expected to result in adverse effects (EVM, 2003a). The EVM noted that intakes greater than 1,000 mg/person/day may result in adverse gastrointestinal effects, but that such effects are associated with supplemental, bolus doses of vitamin C rather than vitamin C intake from food, and therefore, did not propose a guidance level. Furthermore, the

Scientific Panel of the EFSA and the EVM both reported that there was no consistent evidence in support of increased oxalate excretion or kidney stone formation upon high vitamin C intakes.

The EFSA's Scientific Panel on Food Additives, Flavorings, Processing Aids, and Materials in Contact with Food specifically evaluated the safety of calcium ascorbate with a content of threonate (*i.e.*, Ester-C® containing calcium threonate) as a source of ascorbic acid for use in food supplements (EFSA, 2007). Upon review of the data, the scientific panel noted that calcium ascorbate with a content of threonate, calcium threonate, and L-threonic acid all possess low oral toxicity and are not mutagenic. Although long-term studies and studies on the carcinogenicity and reproductive and developmental toxicity of the ingredient were not presented, the panel concluded that such studies were not required considering that calcium ascorbate with a content of threonate dissociates to substances that are physiologically present in the human body (ascorbate, calcium, and threonate) and that the panel had previously evaluated the safety of ascorbic acid and calcium. The panel concluded that the additional exposure to calcium and threonate as a result of use of the ingredient is not of safety concern, and that the use of calcium ascorbate containing up to 2% threonate as a source of ascorbic acid in food supplements is further not of safety concern.

2. Calcium

Briefly, with regards to safe levels of calcium intake, the IOM previously had set a UL of 2,500 mg/person/ day from all sources (*e.g.*, food and supplements) for all age groups, including pregnant or lactating women, based on the risk for hypercalcemia and renal insufficiency at intakes ranging from 4,000 to 5,000 mg of calcium/person/day and greater (IOM, 1997). A UL for infants aged 0 to 12 months could not be established due to insufficient data.

The IOM was subsequently asked to review the current data and in 2011 published updated ULs (IOM, 2011). For infants, new data were available regarding calcium excretion that suggested that infants can tolerate intakes of up to approximately 1,750 mg/day. Thus, a no-observed-adverse-effect level (NOAEL) of 1,750 mg/day was established. For infants 0 to 6 months of age, an uncertainty factor of 2 was applied to account for body weight differences and the UL was set at 1,000 mg/day. The UL for infants aged 7 to 12 months was set at 1,500 mg/day on the basis that an increased capacity to handle calcium would accompany increased body size. The UL for children aged 1 through 8 was not changed and remains at 2,500 mg/day, whereas the UL for children aged 9 to 13 years and adolescents aged 14 to 18 years, including pregnant and lactating adolescents, was raised by 500 mg/day to 3,000 mg/day to account for the increase in bone accretion and likely accompanying increases in tolerated intakes. For adults, although the IOM recognized that hypercalcemia was an adverse outcome, they noted that it was a disease state and they did not consider it appropriate for the derivation of ULs for the normal, healthy population. Kidney stone formation was selected as the indicator, and an UL of 2,000 mg/day was set for adults aged 51 to 70 and greater than 70 years based on increased risk of kidney stone formation at higher intakes. It was noted by the IOM

committee that "intakes of calcium from food do not readily result in excess intakes and are not associated with adverse effects; rather, the adverse effects appear to be a function of calcium supplementation added to baseline intake" (IOM, 2011). Although kidney stone formation does occur in younger adults, the IOM committee noted that it does not appear to be correlated with calcium supplement use, and thus established an UL of 2,500 mg/day for adults, including pregnant and lactating women, aged 19 to 30 and 31 to 50 years using an interpolation approach based on the mid-point between the UL for adolescents and persons greater than 50 years of age.

The European Commission's Scientific Committee on Food (SCF) established a UL of 2,500 mg/person/day from all sources for all age groups, including pregnant or lactating women, based on no adverse effects observed at this intake level in human studies (SCF, 2003). A UL could not be derived for infants, children, and adolescents based on insufficient data. Furthermore, the EVM determined that a Safe Upper Level for calcium could not be established based on insufficient data from animal and human studies; however, the EVM proposed that, for guidance purposes only, supplemental calcium intake of doses up to 1,500 mg/person/day would not be expected to result in adverse effects (EVM, 2003b). Established ULs and guidance levels for calcium are well in excess of the levels (all-user 90th percentile estimated daily intake of 74 mg/person/day) provided from the intended use levels of Ester-C®.

3. Calcium L-Threonate

Calcium L-threonate, the calcium salt of L-threonic acid, was recently evaluated by the EFSA's Scientific Panel on Food Additives and Nutrient Sources added to Food (EFSA, 2008). The Scientific Panel noted that calcium L-threonate has low oral acute toxicity, and from subchronic toxicity studies, identified a NOAEL of 4,000 and 1,000 mg/kg body weight/day in rats and dogs, respectively. The NOAELs are equivalent to 516 mg calcium/kg body weight/day and 3,484 mg L-threonate/kg body weight/day in rats and 129 mg calcium/kg body weight/day and 871 mg L-threonate/kg body weight/day in dogs. The panel also noted that calcium L-threonate was not genotoxic, and that carcinogenicity studies were not required considering that L-threonate is physiologically present in the human body. Reproductive and developmental toxicity studies reviewed demonstrated no maternal or reproductive toxicity or teratogenicity in mice. The panel concluded that the use of calcium L-threonate in food supplements is not of safety concern at levels providing 1,350 to 2,700 mg of L-threonate (L-threonic acid) per person per day, or 22.5 to 45 mg/kg body weight/day, given the large margin of safety between the estimated human exposure to L-threonate and the NOAELs reported in the subchronic toxicity studies.

In addition, the EFSA's Scientific Panel on Food Additives, Flavorings, Processing Aids, and Materials in Contact with Food specifically evaluated the safety of calcium ascorbate with a content of threonate (*i.e.*, Ester-C® containing calcium threonate) as a source of ascorbic acid for use in food supplements (EFSA, 2007). Following review of the toxicological data, the panel concluded that the additional exposure to calcium and threonate as a result of use of the

ingredient is not of safety concern, and that the intended use levels of calcium ascorbate in the range of 646 to 1,292 mg (providing 500 to 1,000 mg of ascorbic acid), containing up to 2% threonate, as a source of ascorbic acid in food supplements is further not of safety concern. The conclusion of these safety assessments and the studies described above support the safe use of calcium L-threonate as a starting material in the manufacture of Ester-C® and as a component of the Ester-C® ingredient.

4. Hydroxymethyl Furanone

JECFA evaluated the safety of hydroxymethyl furanone and concluded that hydroxymethyl furanone does not present a safety concern at current levels of intake when used as a flavoring agent and has specified an ADI of "acceptable[2]" for this compound (JECFA, 2004, 2005). In the toxicological monograph that was prepared for tetrahydrofuran and furanone derivatives, which included toxicity data on hydroxymethyl furanone, it was concluded that high doses of 3-(2*H*)-furanone derivatives were shown to be genotoxic in bacterial and mammalian assays, but failed to result in carcinogenic effects in rats administered doses up to 200 mg/kg body weight/day for 1 to 2 years (JECFA, 2006b). Following review of the available data and given the low intakes of these flavoring agents in the European and U.S. populations, the Committee concluded that it is highly unlikely that tetrahydrofuran or furanone derivatives would pose any significant genotoxic risk to humans under the conditions of use as flavoring agents.

In 2011, the EFSA assessed whether calcium ascorbate with a content of threonate and the by-product hydroxymethyl furanone (*i.e.*, Ester-C® containing calcium threonate and hydroxymethyl furanone) was covered by the previous opinion on calcium ascorbate with a content of threonate (EFSA, 2011). Considering data from a previous assessment of hydroxymethyl furanone within a group of related α,β-unsaturated 3(2)-furanones and using a read across approach, the EFSA concluded that "the residual level of 4-HMF [4-hydroxy-5-methyl-3(2H)-furanone] in calcium L-ascorbate with a content of threonate is unlikely to be a safety concern". They further concluded that "the safety of the proposed uses of the source produced by the new production process can be considered as covered by the existing EFSA Opinion on '*Calcium ascorbate with a content of threonate for use as a source of vitamin C in food supplements*' (EFSA, 2011).

IV.C Absorption, Distribution, Metabolism, and Elimination

1. Calcium Ascorbate and Ascorbic Acid

Following ingestion, calcium ascorbate is hydrolyzed in the stomach and dissociates into its constituent ions, calcium and ascorbate. Gastric acid (hydrochloric acid) in the stomach then converts the ascorbate ions into ascorbic acid. Calcium and ascorbic acid are subsequently

[2] A term used to describe flavouring agents that are of no safety concern at current levels of intake. If an ADI has been allocated to the agent, it is maintained unless otherwise indicated (WHO, 1987).

absorbed from the intestinal tract into the systemic circulation. This is supported by observations in both humans and experimental animals demonstrating a rapid rise in plasma ascorbic acid concentrations following a single oral dose of various calcium ascorbate formulations, including Ester-C® (containing calcium threonate, but not hydroxymethyl furanone).

In humans, ingestion of an acute dose of 1,000 mg of calcium ascorbate resulted in an increase in plasma and serum ascorbic acid levels in as early as 1 hour regardless of the formulation, with peak levels occurring within 2 hours (Johnston and Luo, 1994; Moyad *et al.*, 2008; Pancorbo *et al.*, 2008). However, compared to a formulation containing only calcium ascorbate, the amount of ascorbic acid absorbed from Ester-C® (containing calcium threonate) was greater throughout the first few hours post-ingestion (Pancorbo *et al.*, 2008). The absorption and pharmacokinetic profile of ascorbic acid from Ester-C® instead is similar to that from ascorbic acid (Johnston and Luo, 1994; Moyad *et al.*, 2008; Pancorbo *et al.*, 2008). With regards to absorption, ascorbic acid is rapidly absorbed from the intestinal tract and into the circulation following oral ingestion (detection within 15 to 30 minutes), with peak plasma levels occurring within 45 minutes to 4 hours depending on the dose (Blanchard *et al.*, 1990; Bluck *et al.*, 1996; Graumlich *et al.*, 1997; Bates *et al.*, 2004; Padayatty *et al*, 2004).

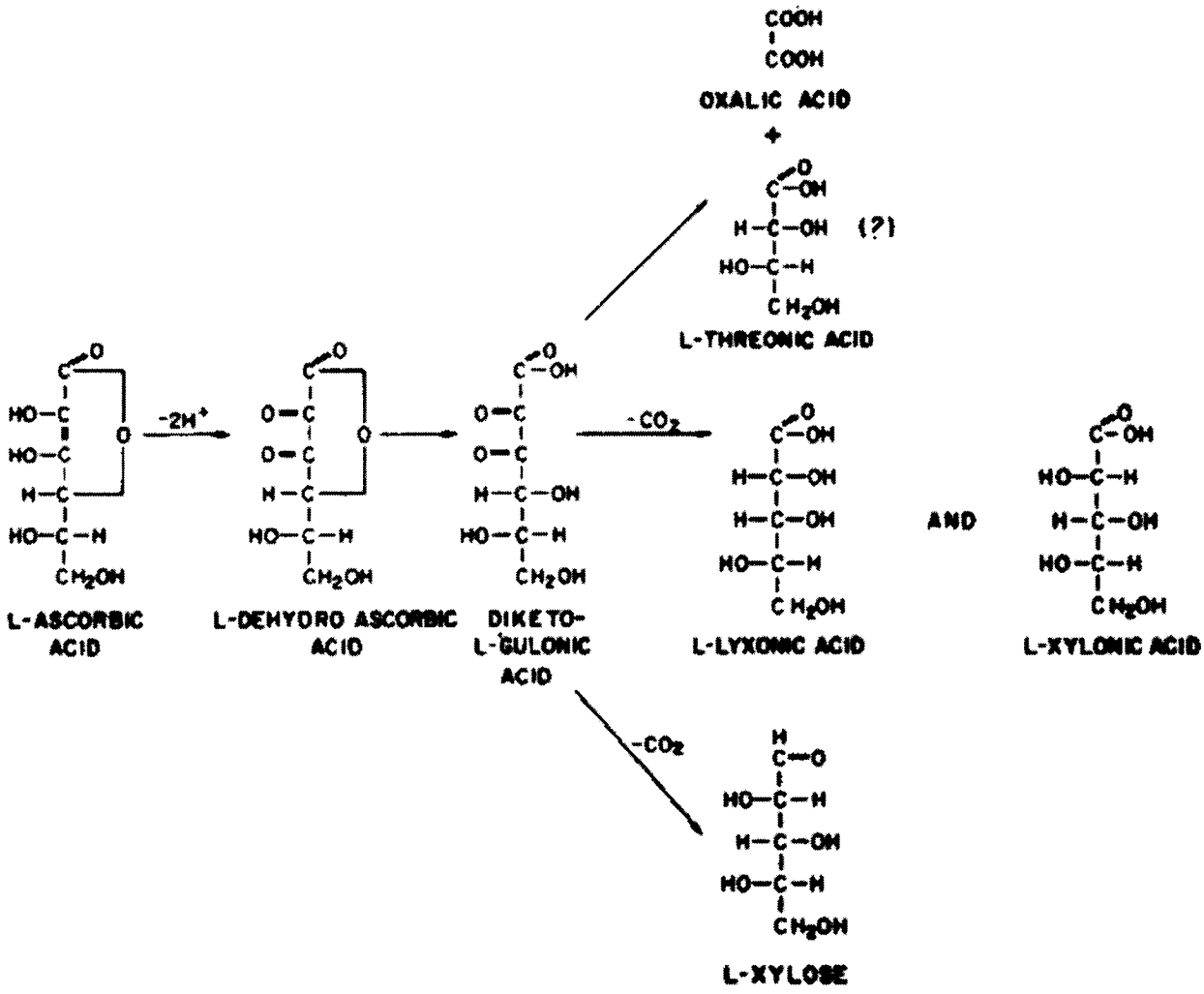
At typical dietary intakes of ascorbic acid (30 to 180 mg/person/day), absorption of the vitamin is efficient with approximately 70 to 90% of the ingested amount being absorbed (Kallner *et al.*, 1979). At these low gastrointestinal concentrations of ascorbic acid, intestinal absorption occurs *via* a sodium-dependent active transport mechanism, which is dose-dependent (Rumsey and Levine, 1998). This mechanism of absorption starts becoming saturated at intakes of approximately 200 mg/person at which point increasing doses result in very little increase in plasma ascorbic acid concentrations (Blanchard *et al.*, 1997; Graumlich *et al.*, 1997; Padayatty *et al*, 2004). At intakes of approximately 1,000 mg/person/day and greater, absorption levels drop to approximately 50% of the ingested amount (Kallner *et al.*, 1979; Blanchard *et al.*, 1997).

The rapid absorption of ascorbic acid from Ester-C® observed in humans is supported by studies conducted in rats and dogs. For instance, in male Sprague-Dawley rats gavaged with 27.4 mg/kg body weight of Ester-C®, continually increasing plasma ascorbic acid concentrations were detected during the 20 to 80 minutes post-administration (Bush and Verlangieri, 1987). Elevated ascorbic acid levels were maintained at 208 minutes (3.5 hours) post-calcium ascorbate administration. The rate of absorption of ascorbic acid from calcium ascorbate was determined to be 0.099 µg/minute, which was more rapid than that from 20 mg/kg body weight of L-ascorbic acid (0.026 µg/minute). In dogs, plasma ascorbic acid levels increased within the first hour post-administration of oral capsules providing 15 or 50 mg/kg body weight of Ester-C® (Wang *et al.*, 2001). Maximal plasma ascorbic acid levels were reached within 3 hours. In contrast to findings in rats, a similar pharmacokinetic profile was obtained following ingestion of equivalent doses of ascorbic acid.

Ascorbic acid is widely distributed throughout the human body. The highest concentrations of ascorbic acid have been reported in the adrenal and pituitary glands, with smaller levels observed in the liver, spleen, lungs, kidneys, testes, thyroid, heart, skeletal muscle, brain, pancreas, and eyes (Hornig, 1975). Distribution of ascorbic acid to tissues is a saturable process, with intracellular stores becoming saturated at doses of 200 mg/person and greater (Graumlich *et al.*, 1997). The biological functions of ascorbic acid are based on its reducing properties, and therefore, ascorbic acid functions as a water soluble oxidation-reduction system and as a scavenger for reactive oxygen species, as well as a co-factor for various enzymes requiring its reducing power (Tolbert, 1985).

The metabolism of ascorbic acid involves oxidation to dehydroascorbic acid, which can be reduced back to ascorbic acid or further oxidized to diketogulonic acid, which, in turn, is oxidized to oxalic acid, threonic acid, xylose, xylonic acid, and lyxonic acid (Figure IV.B-1) (Burns, 1975). Ascorbic acid also may undergo conjugation with sulfate to form ascorbate-2-sulfate, which is excreted in the urine (Baker *et al.*, 1975; Tolbert, 1985). Metabolites of ascorbic acid are excreted in the urine. Additionally, in humans, intake of an ascorbic acid supplement at escalating doses of up to 2,000 mg/day for 10 days resulted in an increase in urinary hydroxymethyl furanone levels (Moyad *et al.*, 2009). Similar results were observed following intake of Ester-C® at doses providing up to 2,000 mg/day of vitamin C. This finding suggests that hydroxymethyl furanone is formed as a result of ascorbic acid metabolism in humans.

Figure IV.C-1 Metabolism of ascorbic acid to urinary metabolites (from Burns, 1975)



In humans, plasma and serum levels of ascorbic acid begin to decline at approximately 3 to 4 hours following ingestion of an acute dose of calcium ascorbate or ascorbic acid (Graumlich *et al.*, 1997; Moyad *et al.*, 2008; Pancorbo *et al.*, 2008). Elimination of ascorbic acid is gradual over the following 20 hours and the profile is similar among different sources of ascorbic acid (*i.e.*, between Ester-C®, other calcium ascorbate formulations, and ascorbic acid) (Moyad *et al.*, 2008; Pancorbo *et al.*, 2008). Specifically, the elimination half-life for ascorbic acid has been reported as being 5 hours following ingestion of 500 mg ascorbic acid (Blanchard *et al.*, 1990). Ascorbic acid undergoes renal tubular absorption, and thus, very little unchanged ascorbic acid is excreted in the urine with intakes of up to approximately 80 mg/day (Kallner *et al.*, 1979; Blanchard *et al.*, 1997). At larger intakes, however, urinary excretion of unchanged ascorbic acid increases proportionally as a result of saturation of tubular reabsorption (Kallner *et al.*, 1979; Blanchard *et al.*, 1990, 1997). In rats, the average time of initial appearance of ascorbic

acid in the urine has been reported as approximately 200 minutes following oral intake of 27.4 mg/kg body weight of Ester-C® (Bush and Verlangieri, 1987). In comparison, the average time of initial appearance of ascorbic acid in the urine following oral intake of 20 mg/kg body weight of L-ascorbic acid was reported as approximately 100 minutes. In dogs, the majority of absorbed ascorbic acid from Ester-C® was eliminated within 12 hours, similar to ascorbic acid (Wang *et al.*, 2001).

2. Calcium

Dissociation of calcium ascorbate is further evidenced by the rapid rise in plasma calcium concentrations following a single oral dose of calcium ascorbate. In rats, peak plasma calcium levels have been shown to occur at 1 hour post-administration of 5 to 25 mg of calcium as calcium ascorbate, followed by a gradual decline over the following 33 hours (Tsugawa *et al.*, 1999; Cai *et al.*, 2004). Calcium is absorbed from the duodenum and occurs *via* a vitamin D-dependent active transport mechanism at relatively low dietary intakes (Bronner, 2003). Following entry into enterocytes, calcium is transported across the cell by binding to the cytosolic transport protein calbindin and is extruded from the cell into the circulation *via* an adenosine triphosphatase pump. Conversely, when dietary calcium intakes are high, calcium is absorbed paracellularly in the ileum by diffusion through tight junctions (Bronner, 2003). Following absorption, calcium is distributed predominantly to mineralized tissues, such as bone and teeth, where it is present as calcium phosphate (IOM, 1997). Ninety-nine percent (99%) of the body's calcium stores are found in these tissues and the remaining 1% occurs in blood, extracellular fluid, muscle, and other tissues (IOM, 1997). Calcium is excreted from the body by both urinary and fecal routes (Cashman, 2002; Cai *et al.*, 2004; Bhatia, 2008).

3. Threonic Acid

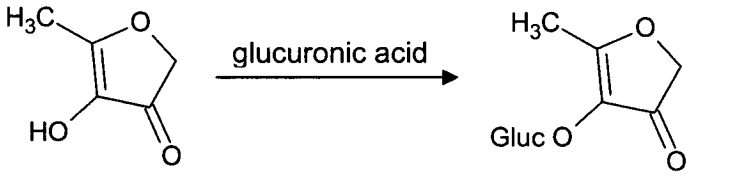
Whether threonic acid is absorbed following ingestion of threonic acid itself is unknown; however, threonic acid occurs in the human body as a metabolite of ascorbic acid (Burns, 1975; Deutsch *et al.*, 1999; Harding *et al.*, 1999). Threonic acid is excreted unchanged in urine in both animals and humans (Knafo *et al.*, 2005; Wang *et al.*, 2006). No other information on the metabolic fate of threonic acid *in vivo* was identified in the publicly available literature.

4. Hydroxymethyl Furanone

Although information on the metabolic fate of hydroxymethyl furanone is limited, furanone derivatives are expected to be readily absorbed and rapidly eliminated in urine. For instance, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF) and 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone (EHMF) were detected in plasma samples from mice as early as 15 minutes following a single gavage administration of 1,000 mg/kg body weight of either compound (Hiramoto *et al.*, 1998). Peak plasma levels of EHMF and DMHF were attained between 15 and 45 minutes post-administration, and both compounds were eliminated from the blood by 2 hours. DMHF is

excreted in urine as a glucuronide conjugate as was observed in human volunteers following consumption of strawberries naturally containing DMHF (Roscher *et al.*, 1997). Unconjugated (free) DMHF was not detected in urine. The amount of DMHF ingested was approximately 50 to 180 mg/person within an 8-hour period, 59 to 94% of which was excreted in urine within 24 hours. Considering the structural similarity to DMHF, hydroxymethyl furanone is expected to be rapidly absorbed from the gastrointestinal tract, metabolized *via* conjugation with glucuronic acid, and subsequently excreted in the urine (Figure IV.C-2).

Figure IV.C-2 Metabolic fate of hydroxymethyl furanone



urinary metabolite

5. Summary

Ester-C® mainly comprises calcium ascorbate, which is hydrolyzed and dissociated in the stomach to calcium and ascorbate. Ascorbic acid and calcium are both rapidly absorbed from the intestinal tract into the systemic circulation. This is supported by observations in both humans and experimental animals demonstrating a rapid rise in plasma ascorbic acid and calcium concentrations following a single oral dose of various calcium ascorbate formulations, including that of Ester-C® (containing calcium threonate, but not hydroxymethylfuranone). The absorption and pharmacokinetic profile of ascorbic acid from ingestion of calcium ascorbate is similar to that from the ingestion of ascorbic acid itself. Absorption of ascorbic acid from the intestinal tract is mediated by a saturable sodium-dependent active transport mechanism, and thus, is most efficient at relatively low typical dietary intake levels. Following absorption, ascorbic acid is distributed throughout the body, with the highest levels reported in the adrenal and pituitary glands. The metabolism of ascorbic acid is mainly oxidative in nature, with initial formation to dehydroascorbic acid. Dehydroascorbic acid can be reduced back to ascorbic acid or further oxidized to oxalic acid, threonic acid, xylose, xylonic acid, and lyxonic acid. Conjugation of ascorbic acid with sulfate, as well as the formation of hydroxymethyl furanone, also have been reported to occur. The bulk of an absorbed dose of ascorbic acid is eliminated rapidly from the circulation and reabsorbed by the kidneys or excreted in the urine at higher intake levels.

The absorption of calcium from the intestinal tract also is mediated by a saturable active transport mechanism, which is vitamin D-dependent. In addition, at high dietary intake levels, calcium is absorbed paracellularly by diffusion through tight junctions. Following absorption,

calcium is distributed predominantly to mineralized tissues, such as bone and teeth, and is excreted from the body by both urinary and fecal routes.

In addition to ascorbic acid and calcium, Ester-C® contains minimal amounts of threonic acid and hydroxymethyl furanone. Although the absorption of threonic acid from the intestinal tract is not documented, threonic acid does occur in the human body as a physiological metabolite of ascorbic acid and undergoes excretion by the urinary route. Information on hydroxymethyl furanone also is limited; however structurally similar furanones are readily absorbed, metabolized *via* conjugation with glucuronic acid, and eliminated in the urine.

IV.D Toxicological Studies

1. Acute Toxicity Studies

Ester-C®, containing calcium threonate but not hydroxymethyl furanone, was assessed for acute toxicity, a summary of the results of which were made generally available *via* the EFSA's publication on the Opinion of the Scientific Panel on Food Additives, Flavorings, Processing Aids, and Materials in Contact with Food on the use of calcium ascorbate with a content of threonate as a source of vitamin C in food supplements (EFSA, 2007). As summarized by the EFSA, Ester-C® did not induce signs of toxicity when orally administered to rats at a single dose of 7,500 mg/kg body weight by gavage in a water solution or in corn oil. The rats were observed for 14 days following Ester-C® administration. Further details were not provided.

Similarly, neither calcium threonate nor the hemicalcium salt of L-threonic acid induced signs of toxicity when orally administered to rats at a single dose of 5,000 mg/kg body weight by gavage in a water solution (rats were observed for 14 days following administration) (EFSA, 2007). Calcium L-threonate also was not acutely toxic to Kunming mice or Wistar rats (10 per sex) when orally administered by gavage at a single dose of 40 or 32 g/kg body weight, respectively (animals were observed for 10 days following administration) (Gao *et al.*, 1997a) as summarized by the EFSA's Scientific Panel on Food Additives and Nutrient Sources in their opinion on the use of calcium L-threonate as a source of calcium in food supplements (EFSA, 2008). Further details of the acute studies were not provided.

2. Repeated Dose Studies

Subchronic and chronic studies conducted on Ester-C®, calcium L-threonate, threonic acid, and hydroxymethyl furanone are summarized in Table IV.D-1. These studies also are described below in Sections 2.1 through 2.3.

Table IV.D-1 Summary of Subchronic and Chronic Toxicity Studies on Ester-C®, Hydroxymethyl Furanone, Calcium Threonate, and Threonic Acid

Species (Sex; strain; n)	Study Duration	Route of Administration	Dose (mg/kg bw/day)[a]	Tested Parameters	Results[b,c]	NOAEL (mg/kg bw/d)[d]	Reference
Ester-C®							
Rat [mutant Wistar (ODS); 5/group]	24 days	Oral (*via* drinking water)	0.08 mg/mL (provided in drinking water)	Body weight gain, signs of gross toxicity, morbidity, scorbutic parameters (hemorrhage, reduced exploratory behavior, piloerection, reduced mobility, dysbasia, and ataxia)	No signs of morbidity. Normal body weight gain. Low scores for scorbutic parameters.	N/A	Verlangieri *et al.* (1991)
Rat (M, F; Hsd:SD®; 10/sex/group)	At least 28 days	Oral (*via* diet)	0 (control), 1,140, 2,860 or 5,710	Mortality, signs of gross toxicity, behavioral changes, ophthalmology, food consumption, body weight, clinical chemistry, hematology, serology assessments, gross necropsy, and histopathology	No significant differences.	5,710	Eurofins Product Safety Laboratories (2007)
Threonic Acid							
Guinea-pig (M; albino Dunkin-Hartley; 10/group)	4 days	Oral (route not specified)	5 of ascorbic acid with 0 (control) or 100 threonic acid	Food intake, body and organ weights, and tissue ascorbic acid concentrations	↓ Ascorbic acid concentrations in liver, adrenal glands, and testes.	N/A	Thomas and Hughes (1983)
	28 days			Food intake, body and organ weights, tissue ascorbic acid concentrations, hematology (PCV and Hb), plasma cholesterol, ALP, ALT, and AST levels	↓ Ascorbic acid concentrations in liver and spleen.	N/A	
	Life span		0 (control) or 100	Mean life-span period	↓ Mean life-span (by 2.4 days) attributed to ascorbic acid deficiency.	N/A	

Table IV.D-1 Summary of Subchronic and Chronic Toxicity Studies on Ester-C®, Hydroxymethyl Furanone, Calcium Threonate, and Threonic Acid

Species (Sex; strain; n)	Study Duration	Route of Administration	Dose (mg/kg bw/day)[a]	Tested Parameters	Results[b,c]	NOAEL (mg/kg bw/d)[d]	Reference
Calcium Threonate							
Mouse (M, F; Tuck No. 1; 65/sex/group)	Life span	Oral (*via* diet)	0 (control), 75, or 300	Mean life-span and food intake.	No significant differences.	300	Thomas and Hughes (1985)
Rat (M; albino Wistar; 10/group)	120 days		0 (control) or 1,000	Food intake, growth rate, organ weights, plasma cholesterol and cytochrome P450 levels, and hematology (PCV and Hb)	↓ Relative liver weight.	N/A	
Rat (M, F; strain NS; 10 or 15/sex/group)	24 weeks with 2 weeks recovery period	Oral (*via* gavage)	0 (control), 2,000, 4,000, or 6,000	Clinical appearance, body weight, hematology, and clinical chemistry	No mortalities. ↓ Spontaneous motor activity and loose stool on several occasions in some animals [4,000]. ↓ Coagulation time [4,000] Presence of gas and yellow liquid in the intestine [4,000] and thyroid gland accretion [M: 4,000]; effects not observed after the recovery period. No other changes in final body weights, hematology, clinical chemistry, and histopathology. Changes observed were attributed by authors to high calcium intake.	4,000[e]	Gao *et al.* (1998)
Dog (M, F; strain NS; 2 to 3/sex/group)	24 weeks with 2 weeks recovery period	Oral (route not specified)	0 (control), 1,000, 2,000, or 3,000	Mortality, general appearance, psychomotility, appetite, feed and water intake, urine and fecal analysis, hematology, clinical chemistry, and electrocardiograms	Slight hyperplasia of the thyroid gland with histopathological findings [2,000 and 3,000]; effects not observed after recovery period and attributed to high calcium intake.	1,000[e]	Zhao *et al.* (1997)

Table IV.D-1 Summary of Subchronic and Chronic Toxicity Studies on Ester-C®, Hydroxymethyl Furanone, Calcium Threonate, and Threonic Acid

Species (Sex; strain; n)	Study Duration	Route of Administration	Dose (mg/kg bw/day)[a]	Tested Parameters	Results[b,c]	NOAEL (mg/kg bw/d)[d]	Reference
Hydroxymethyl Furanone							
Mice (F; ICR; 28 to 30/group) with benzo[a]pyrene-induced forestomach neoplasia	17 weeks	Oral (*via* diet)	0 (control), 4.3, 8.7, or 13.0	Body weight, food intake, development of forestomach neoplasia	↓ Number of tumors per mouse and tumors per tumor bearing mouse [8.7, 13.0]. ↑ Food intake [13.0] No other changes in food intake or body weights.	13.0	Kataoka *et al.* (1997)
Rat (M, F; strain NS; 3/sex/group, except 6/sex/group in control group)	4 weeks	Oral (*via* diet)	0 (control), 7, 18, 36, 73, 109, or 146	Body weight, food and water consumption, food utilization, hematology, and necropsy	↑ Erythrocyte volume fraction [M: 18, 36, 73, 109; F: 18, 36]. ↑ Total leukocyte counts [F: 36]. ↑ Relative liver weights [F: 146]	146	Munday and Kirkby (1971a)
Rat (M, F; Colworth Wistar; 6/sex/group, except 12/sex/group in control)	6 weeks	Oral (*via* diet)	0 (control), 7, 73, or 146	Clinical chemistry, urinalysis, macroscopic and microscopic examinations, and absolute and relative weights	↑ Relative liver weights [F: 73, 146] Changes in relative organ weights were sporadic and not dose-dependent. No significant differences in gross and histopathological examination of the liver and other organs.	146	Munday and Kirkby (1971b)
Rat (M, F; Colworth Wistar; 8/sex/group)	13 weeks	Oral (*via* diet)	0 (control), 7, 18, 36, 73, 109, or 146	Body weight, food and water intake, food utilization, urinalysis and clinical chemistry at Week 13 [18, 36, 109], hematology at Weeks 6 and 13 [7, 73, 146], gross necropsy, histopathological examination, and absolute and relative weights	↓ Food intake [109]. No compound-related adverse effects in urinalysis or clinical chemistry. ↓ Erythrocyte volume fraction at Week 6 [7, 146]. ↑ Leukocyte count Week 6 [146]. ↑ Water intake [M: 18; F: 36]. ↑ Absolute kidney weights [M, F: 73, 146].	146	

Table IV.D-1 Summary of Subchronic and Chronic Toxicity Studies on Ester-C®, Hydroxymethyl Furanone, Calcium Threonate, and Threonic Acid

Species (Sex; strain; n)	Study Duration	Route of Administration	Dose (mg/kg bw/day)[a]	Tested Parameters	Results[b,c]	NOAEL (mg/kg bw/d)[d]	Reference
					↑ Relative kidney weights [M: 73; F: 73, 146]. ↑ Absolute and relative liver weights [M, F: 7, 73, 146]. No evidence of histopathological changes in the liver or of other organs.		
Rat (M, F; Colworth Wistar; 4/sex/group)	52 weeks	Oral (*via* diet)	0 (control), 7, 18, 36, 73, 109, or 146 for 13 weeks and 146 from Weeks 15 to 52	Body weight, food and water consumption, food utilization, general health, survival, hematology, macroscopic and microscopic examination, and absolute and relative organ weights (liver, spleen, heart, kidneys, brain, adrenals, pituitary, thyroid, and testes)	No compound-related effects on general health and survival. Lesions (subcutaneous sarcoma, chloroma, pituitary adenoma, and parafollicular thyroid adenoma) were observed in treated and control rats; however, these findings have been reported in previous studies in control rats of the same strain, and therefore, were not compound-related. No significant differences in final mean body weights, absolute and relative organ weights, hematology, and macroscopic examination.	146	Munday and Kirkby (1973)

↓ = decrease/decreased; ↑ = increase/increased; ALT = alanine aminotransferase; ALP = alkaline phosphatase; AST = aspartate aminotransferase; b/w = body weight; F = females; Hb = hemoglobin; M = males; N/A = not applicable; NOAEL = No-Observed-Adverse-Effect Level; NS = not specified; ODS = Osteogenic Disorder Shionogi; PCV = packed cell volume.

[a] Unless otherwise indicated.

[b] Unless otherwise stated, all reported effects are those that were significantly different from the control group. Non-significant effects are not reported.

[c] Numbers in parentheses represent dose levels at which the effects were observed.

[d] A NOAEL was derived based on the conclusions of the authors.

[e] As concluded by the EFSA's Scientific Panel on Food Additives and Nutrient Sources.

2.1 Ester-C®

The oral toxicity of Ester-C®, containing both calcium threonate and hydroxymethyl furanone, was assessed in a 28-day subchronic toxicity study in rats (Eurofins Product Safety Laboratories, 2007). The study was conducted in compliance with the Organisation for Economic Co-Operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (OECD, 1998), with the exception that the serology analysis was not conducted under GLP compliance. The study also was conducted in accordance with the OECD Guidelines for Testing of Chemicals Test No. 407 (OECD, 2008) and with *U.S. FDA Redbook 2000* (U.S. FDA, 2003). Hsd:SD® rats (10/sex/group) were administered diets containing 0 (control), 20,000 (low-dose), 50,000 (mid-dose), or 100,000 (high-dose) ppm of Ester-C® for at least 28 days. Targeted dietary intake levels of Ester-C® were 0, 1,140, 2,860, and 5,710 mg/kg body weight/day, respectively. At dietary concentrations of 20,000, 50,000, and 100,000 ppm, males consumed mean daily doses of 1,638, 4,106, and 8,331 mg Ester-C®/kg body weight/day, respectively. In females, mean daily intakes of 1,669, 4,320, and 8,744 mg Ester-C®/kg body weight/day were achieved at dietary concentrations of 20,000, 50,000, and 100,000 ppm, respectively

All animals survived the course of the study. Several mid- and high-dose males and mid-dose females exhibited transient soft feces, which were considered as non-adverse due to recovery shortly thereafter. Ophthalmoscopic examination of both eyes of all animals was unremarkable.

Significant reductions in mean daily food consumption was reported in the mid and high-dose males during Week 1 compared to controls, although not significant, trends towards reduced body weights in the high dose males were observed on Week 1. This effect was likely related to the bitter taste of ascorbic acid at high dietary concentrations. No significant differences in mean daily food consumption were reported for females. During Weeks 1 through 4 no significant between group differences in mean weekly body weights were reported for either sex.

Compared to controls, clinical chemistry, urinalysis, and hematology results revealed a number of statistically-significant sporadic changes that were deemed incidental and toxicologically insignificant as they were not dose-dependent and/or were observed in one sex only. The following are the changes that were observed:

- Clinical chemistry results revealed decreased sorbital dehydrogenase (SDH) and total protein levels in mid-dose males and decreased cholesterol and increased inorganic phosphorus levels in high-dose males. Decreased globulin levels were reported in male groups fed Ester-C® and decreased sodium levels were reported in female groups fed Ester-C®. In females, chloride levels were decreased in the mid-dose group. The only consistent change observed among both sexes was an increase in blood urea nitrogen

(BUN) levels in high-dose males and females, but this effect was not dose-dependent. In addition, the results of serology examinations were unremarkable.

- Urinalysis results revealed an increase in pH in high-dose males and a decrease in total protein levels in mid-dose females. Crystals in the urine were reported for 1 female of the high-dose group; however due to low incidence and severity, the authors considered this finding to be toxicologically insignificant.

- Hematological data showed a slight increase in red blood cell distribution width in high-dose males; however, this change was not accompanied by any other hematological changes and as such was not considered to be toxicologically significant. Reactive lymphocytic involvement was reported in 1 female in the mid-dose group and neutrophil banding was reported in 1 female in the low-dose group, which is a possible indicator of granulocytic immaturity; however, the authors considered these findings to be not toxicologically significant, since they were each reported in 1 female only. No effects on coagulation parameters were detected.

Compared to control rats, the following statistically-significant changes in organ weights were observed: increased absolute uterine weight in low-dose females, decreased relative ovary and heart weights in mid-dose females, and increased relative epididymis weight in high-dose males. Changes in organ to brain weight ratios were unremarkable; however, decreased kidney to brain weight in high-dose males was reported. These changes in organ weights were sporadic and not accompanied by relevant clinical or histopathological findings, and thus, were considered toxicologically insignificant. Similarly, macroscopic examination upon necropsy and microscopic examination of tissues/organs did not reveal any compound-related abnormalities; incidental findings of enlarged inguinal and iliac lymph nodes observed in 1 female from each of the mid- and high-dose groups were considered toxicologically insignificant as they also were observed in control females and were not correlated with changes in clinical or histopathological data. Based on the results and under the conditions of this study, the authors concluded the NOAEL for Ester-C® to be 100,000 ppm or 10% in the diet for male and female rats, which is equivalent to 8,331 and 8,744 mg/kg body weighty/day, respectively, the highest dose tested.

One study involving repeat administration of Ester-C® was identified in the available literature that included measurement of parameters relevant to safety. The Ester-C® used in this study contained calcium threonate but not hydroxymethyl furanone. Groups of 5 mutant Wistar rats (Osteogenic Disorder Shionogi rats) (sex not specified) were provided 0.08 mg/mL of Ester-C® or 0.06 mg/mL ascorbic acid in distilled drinking water (0.08 mg of Ester-C® and 0.06 mg of ascorbic acid are equal in ascorbate activity equivalents) over a period of 24 days (Verlangieri *et al.*, 1991). Fluid intake and body weights were recorded 3 days per week and rats were rated on a scorbutic (*i.e.*, ascorbic acid depletion-induced scurvy) rating scale (a total score of 1 is considered "least scorbutic" and a total score of 19 is considered "most scorbutic"). This scale was designed to assess the severity of scurvy-induced symptoms, such as hemorrhage,

reduced exploratory behavior, piloerection, reduced mobility, dysbasia, and ataxia. Administration of Ester-C® did not result in overt toxicity as rats administered Ester-C® displayed normal, continuous body weight gain during the course of the study and no signs of morbidity were observed. Rats of the Ester-C® group also displayed very low scores for scorbutic parameters: mean scores of 0.7, 0.4, and 0.3 in the Ester-C® group were reported on Days 5, 14, and 24, respectively, compared to 1.0, 1.4, and 1.9 in ascorbic acid group, respectively.

Together, the results of the animal studies conducted with Ester-C® demonstrate that repeated oral administration of high doses of Ester-C® does not produce adverse effects in rats at dietary intakes of up to approximately 8,000 mg/kg body weight/day, and corroborate the safety of Ester-C® under the intended conditions of use.

2.2 Calcium L-Threonate and Threonic Acid

Ester-C® is manufactured with the use of L-threonic acid in the form of the calcium salt (calcium L-threonate) and also occurs in the final ingredient. However, neither calcium L-threonate nor L-threonic acid currently hold GRAS status, and therefore, a search of the publicly available literature was conducted for data pertaining to the safety of calcium threonate and threonic acid in order to provide a complete safety assessment of Ester-C®. Two studies were identified in which the potential toxicity of threonic acid was investigated and are presented below.

In a study conducted on threonic acid, male albino Dunkin-Hartley guinea pigs (10/group) were administered threonic acid at a dose of 100 mg/kg body weight/day in the form of an oral supplement along with a maintenance dose of ascorbic acid of 5 mg/kg body weight/day for periods of 4 or 28 days (Thomas and Hughes, 1983). Control animals were administered 5 mg ascorbic acid/kg body weight/day only. Prior to this experimental period, all animals received a 1% ascorbic acid solution *via* drinking water for 3 days to produce tissue saturation with ascorbic acid, followed by a 10-day period of ascorbic acid depletion for the purpose of producing a uniform basal concentration of ascorbic acid in all animals. Body weight, food intake, and absolute organ weights (liver, adrenals, spleen, testes, and kidneys) as well as the ascorbic acid concentration of these organs were assessed for all animals. Hematological parameters [packed cell volume (PCV) and hemoglobin] and clinical chemistry [plasma cholesterol, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST)] were assessed following the 28-day period. No significant differences in food intake, initial and final body weights, or organ weights were reported between threonic acid-treated and control guinea pigs. A significant decrease in the ascorbic acid concentration of the liver, adrenal glands, and testes was reported in animals administered threonic acid for 4 days and in the liver and spleen of animals given threonic acid for 28 days compared to controls. No significant differences in hematology parameters or plasma cholesterol levels were observed between groups. In addition, plasma levels of indicators of liver toxicity [alkaline (ALK), ALP, and ALT] were similar between threonic acid and control groups. The authors concluded that threonic acid administration induced a significant decrease in the ascorbic acid

concentration of certain organs, and suggested that threonic acid interfered with the metabolism of ascorbic acid.

Following their observations, Thomas and Hughes (1983) conducted an additional experiment to delineate the overall nutritional significance of the fall in ascorbic acid concentration observed in certain organs of guinea pigs administered threonic acid. To this end, ascorbic acid depletion was achieved in guinea pigs (*i.e.*, scorbutic guinea pigs), who were then orally administered either 100 mg/kg body weight/day of threonic acid or no threonic acid (control). The number of animals in each group was not reported. Monitoring of survival rate revealed that the lifespan of scorbutic guinea pigs receiving supplemental threonic acid was significantly reduced compared to those not receiving threonic acid. The mean survival rates were 30.0 days in the threonic acid group and 32.4 days in the control group, a difference of 2.4 days. The authors attributed this effect on survival time to a threonic acid-ascorbic acid interaction and not to toxicity induced by threonic acid in light of the finding that threonic acid had no effect on a number of physiological and biochemical characteristics customarily regarded as being of significance in toxicity studies. The observed effect on survival rate, however, was more likely due to ascorbic acid depletion.

Thomas and Hughes subsequently conducted a 120-day toxicity study on calcium threonate in male albino Wistar rats (Thomas and Hughes, 1985). Groups of 10 rats were fed diets containing either 1% calcium threonate, providing a dose equivalent to approximately 1,000 mg/kg body weight, or a control diet containing no added calcium threonate for 120 days. Diets were adjusted for isocaloricity and calcium content. Food intakes were recorded at intervals during the course of the experiment and body weights were measured daily. Animals were killed after 120 days and organs were removed and weighed and blood was collected for the analysis of hemoglobin, PCV, plasma cholesterol, and cytochrome P-450 levels. A significant, yet slight, decrease in relative liver weight was observed in calcium threonate-fed rats compared to control rats; the toxicological significance of this observation was not discussed. No other significant differences were observed between the 2 groups in food intake, growth rate, absolute and relative organ weights, clinical chemistry results (plasma cholesterol, cytochrome P-450), or hematology (packed cell volume and hemoglobin). A second experiment was conducted in which mice (65/sex/group) were fed diets containing 0.05 or 0.20% calcium threonate, providing doses of approximately 75 and 300 mg/kg body weight/day, respectively, and observed for mortality. Control animals were fed a similar diet without calcium threonate. There was no significant difference in the life span of calcium threonate-fed mice compared to control mice. Based on the results of the study, the authors concluded that there was "an essential lack of toxicity of threonic acid in rats and mice at dietary concentrations very much in excess of any amounts likely to be ingested by humankind".

Recently, the EFSA's Scientific Panel on Food Additives and Nutrient Sources added to food rendered an opinion on the use of calcium L-threonate as a source of calcium in food

supplements (EFSA, 2008). Additional data on the potential toxicity of calcium L-threonate from repeated oral exposure was made available through this publication in summary form and is presented here. Specifically, 2 subchronic studies, 1 conducted in rats and 1 conducted in dogs, are summarized. In the 24-week toxicity study conducted in rats (strain not specified), animals were orally administered calcium L-threonate at doses of 0 (control), 2,000, 4,000, or 6,000 mg/kg body weight/day, 6 days per week, by gavage for a period of 24 weeks (Gao *et al.*, 1998). A 3-week recovery period followed the 24-week administration period. Fifteen (15) rats/sex/group comprised the control and high-dose (6,000 mg/kg body weight/day) groups and 10 rats/sex/group comprised the low and mid-dose groups (2,000 and 4,000 mg/kg body weight/day, respectively). At Week 12, 5 rats of each sex from each of the control and high-dose groups were killed and at Week 24, 7 rats of each sex were killed, while 3 rats of each sex were observed during the 3-week recovery period. Changes in clinical appearance in all animals were observed daily while body weights were measured weekly up to Week 12, and once every second week thereafter. Blood samples were collected at Weeks 12 (the control and high-dose groups only), 24, and 27 and for clinical chemistry and hematological analysis. All animals survived the course of the study. Clinical examinations of the animals revealed decreased spontaneous motor activity and loose stools on several occasions in some animals in the high-dose group. No significant difference in body weight was reported in the low- and mid-dose groups compared to the control group. Significantly reduced body weights were reported for high-dose males and females from Weeks 4 to 8 and Weeks 4 to 22, respectively; however, final body weights were not significantly different from those of control animals. A shorter coagulation time was observed in the high-dose groups compared to the control groups. No other significant differences were observed in the hematology results or in the clinical chemistry parameters assessed. No abnormalities or histopathological changes were observed in low- and mid-dose animals. In high-dose animals, the presence of gas and yellow liquid was observed in the intestines upon gross examination. A mild thyroid gland accretion was observed in high-dose males; however, this finding was not observed after the recovery period. The authors suggested that a high dose of 6,000 mg/kg body weight/day of calcium L-threonate resulted in high calcium intakes, which induced the effects observed in the coagulation time and in the thyroid gland. Based on the results of this study, the Scientific Panel determined the NOAEL for calcium L-threonate to be 4,000 mg/kg body weight/day in rats.

In the toxicity study conducted in hybrid dogs, animals were orally administered calcium L-threonate at doses of 0 (control; n=2/sex), 1,000 (n=2 males, 3 females), 2,000 (n=3 males, 2 females), or 3,000 (n=2/sex) mg/kg body weight/day, 6 days per week (Zhao *et al.*, 1997). A 2-week recovery period followed the 24-week administration period. Data on general appearance, psychomotility, appetite, and feed and water intake, as well as urine and fecal samples were collected daily. Blood samples were collected before administration, after 2, 4, and 6 months, and 2 weeks after the last administration (recovery period) and for clinical chemistry and hematological analysis. Electrocardiograms were conducted prior to administration, after 3 and 6 months, and after the recovery period. Animals (2/group) were

killed at the end of the administration period, and the remaining animals were killed after the recovery period. All animals survived the course of the study. No differences were observed in the measured parameters or at necropsy in the calcium L-threonate groups compared to the control group. Slight hyperplasia of the thyroid gland accompanied by a decrease or absence of gelatinous substance in follicles and cubical or columnar epithelial cells, with some follicles exhibiting exfoliated cells was reported in the mid- and high-dose groups (2,000 and 3,000 mg/kg body weight/day, respectively). These effects were not observed after the recovery period. The authors suggested that the observations in the thyroid gland reported at the mid- and high-dose levels were a result of high calcium intake. Based on the results of this study, the Scientific Panel determined the NOAEL for calcium L-threonate to be 1,000 mg/kg body weight/day in dogs.

Therefore, the low levels of calcium threonate provided by Ester-C® are not anticipated to result in adverse effects in humans.

2.3 Hydroxymethyl Furanone

JECFA previously prepared a toxicological monograph for tetrahydroxyfuran and furanone derivatives, including hydroxymethyl furanone (JECFA, 2006b). Data on the potential toxicity of hydroxymethyl furanone from three studies were made available in this toxicological monograph and are presented below as described in their summaries.

In a study by Munday and Kirkby (1971a), rats (3/sex/group and 6/sex/group in control group; strain not specified) were administered diets containing a meat flavor cocktail at a concentration of 0 (control), 197, 492, 983, 1,966, 2,949, or 3,932 ppm diet for a period of 4 weeks. The cocktail was composed of 74.1% hydroxymethyl furanone. Based on an average daily intake of approximately 0, 10, 25, 49, 98, 147, or 197 mg/kg body weight/day of the flavor cocktail, daily intakes of hydroxymethyl furanone were equivalent to 0, 7, 18, 36, 73, 109, and 146 mg/kg body weight/day, respectively. Body weight, food and water consumption, and food utilization were measured 2 or 3 times per week. At the end of the 4-week period, hematological examination and necropsy were performed. No significant differences were observed in body weights, food and water consumption, or in food utilization in hydroxymethyl furanone groups compared to the control group. A significant increase in erythrocyte volume fraction was reported in males fed diets containing the flavor cocktail at 492, 983, 1,966, or 2,949 ppm and in females fed diets containing the flavor cocktail at 492 or 983 ppm. A significant increase in total leukocyte counts was reported in females fed diets containing the flavor cocktail at 983 ppm compared to the controls. No other hematological effects were reported. The authors concluded that due to the lack of a dose-response relationship, the changes observed were not considered to be biologically significant. Changes in absolute and relative organ weights of the liver, spleen, heart, kidneys, and testes were unremarkable, except for a significant increase in relative liver weights of females at the highest dose tested (3,932 ppm); however, macroscopic examination of the tissues of all animals did not reveal any compound-related abnormalities.

In a follow up study, diets mixed with the same meat flavor cocktail, containing 74.1% hydroxymethyl furanone, were administered to weanling Colworth Wistar rats (8/sex/group) at a concentration of 0 (control), 197, 492, 983, 1,966, 2,949, or 3,932 ppm for a period of 13 weeks (Munday and Kirkby, 1971b). Based on the average daily intake of the flavor cocktail at approximately 0, 10, 25, 49, 98, 147, or 197 mg/kg body weight/day, daily intakes of hydroxymethyl furanone were equivalent to 0, 7, 18, 36, 73, 109, and 146 mg/kg body weight/day, respectively. Body weight, food and water intake, and food utilization were measured weekly. Clinical chemistry and urinalysis were performed on animals fed diets containing the flavor cocktail at 492, 983, or 2,949 ppm (providing 18, 36, and 109 mg hydroxymethyl furanone/kg body weight/day) at study end in Week 13. Hematology was performed on animals fed diets containing the flavor cocktail at 197, 1,966, or 3,932 ppm (providing 7, 73, and 146 mg hydroxymethyl furanone/kg body weight/day) at Weeks 6 and 13. Gross necropsy and histopathological examination were performed on all animals at the end of the 13-week study, and absolute and relative weights also were recorded. An additional 6-week study also was conducted in which rats (6/sex/group and 12/sex/group in control) were fed the diet containing the flavor cocktail at a concentration of 0 (control), 197, 1,966, or 3,932 ppm (providing 0, 7, 73, and 146 mg hydroxymethyl furanone/kg body weight/day). Blood and urine samples were collected for biochemical analysis. Gross necropsy and macroscopic and microscopic examinations were performed on all animals at the end of the 6-week study, and absolute and relative weights also were recorded.

In the 13-week study, a significant decrease in food intake was reported in rats fed the diet containing the flavor cocktail at 2,949 ppm and a significant increase in water intake was reported in males and females fed the diet containing the flavor cocktail at 492 and 983 ppm, respectively, compared to controls. The authors concluded that these changes were not compound-related as they were not dose-dependent. Compared to the control group, no compound-related adverse effects were reported for the urine refractive index, urine glutamic-oxalacetic transaminase activity for kidney function, qualitative urine analysis, and clinical chemistry results in hydroxymethyl furanone groups.

In the 6-week study, hematology results revealed significant increases in the erythrocyte volume fraction in male and female rats fed diets containing the flavor cocktail at 197 and 3,932 ppm, respectively, and significant increases in the leukocyte count in male and female 3,932 ppm groups. In contrast, no significant differences were observed in the hematological results of any group of rats fed diets containing the flavor cocktail for 13 weeks when compared to the controls. The authors considered the changes observed not to be compound-related.

In animals treated for 6 weeks, significantly, yet slightly, increased relative liver weights were reported in females at flavor cocktail concentrations of 1,966 and 3,932 ppm when compared to the controls. Significant, yet sporadic, changes in the relative weights of other organs were reported; however, the authors determined that these changes were not dose-dependent.

Furthermore, gross and histopathological examination of the liver and other organs in rats fed hydroxymethyl furanone for a period of 6 weeks revealed no significant findings. In animals treated for 13 weeks, significantly increased absolute kidney weights were observed in males and females of the 1,966 and 3,932 ppm groups compared to control groups. Relative kidney weights also were significantly increased in males of the 1,966 ppm group and in females of the 1,966 and 3,932 ppm groups compared to the controls; however, histopathology results revealed no pathology in any organs examined. In addition, marginally increased absolute and relative liver weights were observed in males and females fed diets containing the flavor cocktail at 197, 1,966, or 3,932 ppm. These results were deemed incidental by the authors since these changes were not supported by any evidence of histopathological changes in the liver. The authors concluded that there were no compound-related adverse effects.

In a subsequent study by Munday and Kirkby (1973), Colworth Wistar rats (4/sex/group) were administered diets containing a meat flavor cocktail at a concentration of 197, 492, 983, 1,966, 2,949, or 3,932 ppm, providing doses of hydroxymethyl furanone of approximately 7, 18, 36, 73, 109, and 146 mg/kg body weight/day, respectively, for a period of 13 weeks. Due to the lack of organ weight changes observed in the 13-week study described above (Munday and Kirkby, 1971b), all animals were subsequently administered diets containing the flavor cocktail at the highest dose tested (3,932 ppm, equivalent to 146 mg hydroxymethyl furanone/kg body weight/day) from Weeks 15 to 52 (*i.e.*, an additional 37 weeks). No compound-related effects on general health and survival were observed in animals fed diets containing hydroxymethyl furanone. Compared to control rats, no significant differences were reported in the final mean body weights, absolute and relative organ weights (liver, spleen, heart, kidneys, brain, adrenals, pituitary, thyroid, and testes), hematology, or macroscopic examination. Lesions (subcutaneous sarcoma, chloroma, pituitary adenoma, and parafollicular thyroid adenoma) were observed in treated and control rats; however, as these findings have been reported in previous studies in control rats of the same strain, the authors considered these lesions to be not related to the administered diet. Furthermore, the authors concluded that the administration of diets containing the flavor cocktail at a concentration of up to 3,932 ppm (providing approximately 146 mg hydroxymethyl furanone/kg body weight/day) for a period of 1 year resulted in no effects on type, incidence, or time of development of tumors in Colworth Wistar rats.

3. Reproductive and Developmental Toxicity Studies

Studies examining the potential developmental and reproductive toxicity of Ester-C® have not been performed. Developmental and reproductive studies on hydroxymethyl furanone also were not identified in the literature; however, reproductive and developmental toxicity studies have been conducted on calcium L-threonate. Details of these studies were made available *via* the EFSA's publication on the Scientific Panel on Food Additives and Nutrient Sources' opinion on the use of calcium L-threonate in food supplements (EFSA, 2008).

Male and female Kunming mice (20/sex) were orally administered calcium L-threonate in doses of up to 6,000 mg/kg body weight/day by gavage for a period of 60 and 14 days, respectively, prior to mating (Wu *et al.*, 1997a). Pregnant dams continued to receive calcium L-threonate throughout the period of organogenesis and were killed on Gestational Day (GD) 21. Pregnancy rate, number of live and dead fetuses, weight of live fetuses, and the number of implantations and resorptions were recorded. Viable fetuses were examined for external abnormalities, visceral alterations, and skeletal abnormalities. No effects of calcium L-threonate were observed on the body weight and reproductive performance of the F_0 generation. The authors commented that no external, visceral, or skeletal malformations were observed in the fetuses; however, these data were not presented.

In a study examining the potential teratogenic effect of calcium L-threonate, doses of up to 6,000 mg calcium L-threonate/kg body weight/day, were orally administered to groups of 20 pregnant Kunming mice by gavage on GD 6 to 15 (Wu *et al.*, 1997b). Body weights of the dams were recorded at regular intervals. On GD 21, the dams were killed, and the number of implantations, resorptions, and dead and viable fetuses, as well as the weight of viable fetuses were recorded. Viable fetuses were examined for external abnormalities, visceral alterations, and skeletal abnormalities. No compound-related effects were observed in the body weights or clinical appearances of the dams in all dose groups. No significant differences were observed in the number of implantations, resorptions, and dead and viable fetuses, or in the body weights of viable fetuses between groups. The authors commented that no external abnormalities, visceral alterations, or skeletal abnormalities were attributable to the administration of calcium L-threonate; however, the data were not presented. The authors concluded that calcium L-threonate was not teratogenic under the conditions of this study.

Calcium L-threonate at doses of up to 6,000 mg/kg body weight/day was orally administered to groups of 20 pregnant Kunming mice from GD 15 to the end of the weaning period (Post-natal Day 21) (Wu *et al.*, 1997c). Body weights of the dams, duration of gestation, the number of live and dead pups, external abnormalities, and indices of physiological and behavioral development were recorded. The potential effect of calcium L-threonate on reproductive performance also was assessed by examining one-to-one matings of the F_1 generation at 60 days of age within each group. No compound-related effects were observed on the length of gestation, litter size, number of live and dead pups, survival rate, developmental parameters, or reproductive performance of the F_1 generation. Additionally, no significant differences were observed in behavior (net climbing test and coordination test) or mebumal-induced sleeping time between groups.

IV.E Mutagenicity and Genotoxicity Studies

1. *In vitro* Studies

1.1 Ester-C®

The mutagenic potential of Ester-C®, containing calcium threonate and hydroxymethyl furanone, was assessed using a bacterial reverse mutation assay (Ames test), which was designed to meet the current OECD Guideline for Testing Chemicals Test No. 471 (OECD, 1997) (BSL Bioservice Scientific Laboratories GmbH, 2007). The study also was conducted in compliance with OECD Principles of GLP (OECD, 1998). The bacterial reverse mutation assay was conducted using the plate-incorporation and the pre-incubation methods in a battery of standard histidine auxotroph strains of *Salmonella* Typhimurium (strains TA98, TA100, TA1535, and TA1537) and the *Escherichia coli* tryptophan auxotroph tester strain WP2 uvrA. *S.* Typhimurium TA100 and TA1535 were employed for evaluation of base pair substitution and strains TA100 and TA1537 were employed for evaluation of frameshift mutations. *E. coli* WP2 uvrA was used for the detection of base pair substitution mutations. The bacteria were exposed to Ester-C® at concentrations of 31.6, 100, 316, 1,000, 2,500, or 5,000 µg/plate with or without S9 metabolic activation. Dimethylsulfoxide (DMSO) served as the negative control. For assays conducted in the absence of metabolic activation, either sodium azide, 4-nitro-o-phenylene-diamine (4-NOPD), or methyl methane sulfonate (MMS) were used as the positive control agent. For assays conducted in the presence of metabolic activation, 2-aminoanthracene (2-AA) was employed as the positive control. A positive mutagenic response was described as a dose-dependent 2-fold increase in the number of revertant colonies compared to the negative control for strains TA100 and *E. coli* WP2 and a 3-fold increase for strains TA1535, TA1537, and TA98. Ester-C® was not cytotoxic in any strain at any of the concentrations tested in the presence of metabolic activation. However, some cytotoxic effects were observed in the absence of metabolic activation in *S.* Typhimurium, but not in *E. coli.* Using the plate incorporation method, cytotoxicity was observed in *S.* Typhimurium TA98 and TA1537 at concentrations of 2,500 µg/plate and higher. Using the pre-incubation method, cytotoxicity was observed in strain TA98 at concentrations of 316 µg/plate and higher, in strain TA1537 at 31.6 µg/plate and higher, and in strains TA100 and TA1535 at 5,000 µg/plate. Treatment with Ester-C® did not result in a positive mutagenic response compared with the negative control in any strain at any concentration with or without metabolic activation. In contrast, a positive mutagenic response was observed following treatment of bacteria with the positive control agents compared to the negative control, validating the sensitivity of the test system and the activity of the S9 mix. The investigators concluded that Ester-C® was non-mutagenic under the conditions of this study in *S.* Typhimurium TA98, TA100, TA1535, and TA1537 and in *E. coli* WP2 uvrA in either the presence or absence of metabolic activation at concentrations up to 5,000 µg/plate, the highest concentration tested.

1.2 Calcium L-Threonate

Several mutagenicity/genotoxicity tests were conducted with calcium L-threonate, which were discussed in the 2008 EFSA publication. In an *in vitro* mutagenicity study using the bacterial reverse mutation assay (the Ames assay), negative results were reported in *S.* Typhimurium strains TA97, TA98, TA100, and TA102 treated with calcium L-threonate at concentrations of 0 to 5,000 µg/plate in the presence and absence of S9 metabolic activation (Gao *et al.*, 1997b). In addition, calcium L-threonate was reported not to induce chromosomal aberrations in cultured Chinese hamster cells at concentrations of up to 2,500 µg/mL with or without metabolic activation (Gao *et al.*, 1997c).

1.3 Hydroxymethyl Furanone

The mutagenic potential of hydroxymethyl furanone was assessed in a reverse mutation assay using the pre-incubation method in *S.* Typhimurium strains TA100 and TA98 at concentrations of 0 to 5,000 µg/plate with and without S9 metabolic activation (Hiramoto *et al.*, 1996). The concentrations tested were approximately 625, 1,250, 2,500, and 5,000 µg/plate. The authors reported that hydroxymethyl furanone was mutagenic in *S.* Typhimurium TA100, a strain sensitive to oxidative mutagenicity, with and without metabolic activation, but not in strain TA98 (Hiramoto *et al.*, 1996). The number of revertant colonies following treatment of strain TA100 with hydroxymethyl furanone was approximately twice that of background numbers, except at the highest concentration tested (5,000 µg/plate) in the absence of metabolic activation. Under the latter conditions, the increase in revertant colonies was less than 2-fold, possibly due to the increase in cytotoxicity observed. In contrast, the authors reported that hydroxymethyl furanone was non-mutagenic in strain TA98 at cytotoxic concentrations.

The DNA strand-breaking activity of hydroxymethyl furanone also was assessed by Hiramoto *et al.* (1996). Supercoiled plasmid DNA was incubated with 90 to 900 µM hydroxymethyl furanone at pH 7.4 (exact concentrations were not specified). The supercoiled plasmid DNA (form I) was reported to be converted to a nicked open circular form (form II) or to a linear form (form III) by single-strand breaking in a concentration-dependent manner. DNA breaking activity also was shown to be time-dependent when DNA was incubated with 440 µM hydroxymethyl furanone at pH 7.4, but not at pH 4.4. The investigators attributed the differential response to degradation of the 3(2H)-furanone structure, with degradation occurring more rapidly at a relatively neutral pH of 7.4 compared to under more acidic conditions (pH 4.4). The reducing capacity of hydroxymethyl furanone also was tested using Fe(III) as a substrate. The authors reported that the production of Fe(II) ion increased in a concentration-dependent manner. Furthermore, the addition of superoxide dismutase, catalase, hydroxyl radical scavengers, spin-trapping agents, or chelating agents was reported to inhibit single-strand breakage induced by hydroxymethyl furanone. Based on the latter two findings, the authors ascribed the observed effects of hydroxymethyl furanone on DNA strand breakage *in vitro* to oxygen derived radicals.

Contrary to the findings of Hiramoto *et al.* (1996), Kataoka *et al.* (1997) demonstrated that hydroxymethyl furanone is a scavenger of hydrogen peroxide (H_2O_2), a reactive oxygen species. Human polymorphonuclear (PMN) leukocytes were stimulated with 12-*O*-tetradecanoylphorbol-13-acetate to produce reactive oxygen species, and then incubated with either 0 (control), 10, 30, 50, or 100 µM of hydroxymethyl furanone. Compared to the control, cells treated with 50 and 100 µM of hydroxymethyl furanone displayed significantly reduced concentrations of H_2O_2 in the order of 45 and 74%, respectively. Similarly, incubation of arachidonic acid-stimulated PMNs with 50 µM hydroxymethyl furanone was shown to reduce H_2O_2 concentrations by 81% compared to the control. Kataoka *et al.* (1997) also demonstrated that hydroxymethyl furanone, at concentrations of 10^{-4} and $5x10^{-5}$ M *in vitro*, significantly reduced the rate of lipid peroxidation of linoleic acid micelles, which was induced by hydroxy radicals formed from the Fenton reaction (reaction between Fe(II) and H_2O_2). The authors concluded that hydroxymethyl furanone possesses antioxidant properties.

The mechanism of action of hydroxymethyl furanone in producing genotoxic responses in *in vitro* assays stems from its reducing capacity as shown by Hiramoto *et al.* (1996). Hydroxymethyl furanone is readily oxidized in the presence of metals, such as Fe(III), and dissolved oxygen, resulting in the formation of a superoxide radical anion, which can dismutase to hydrogen peroxide (JECFA, 2006b). Therefore, hydroxymethyl furanone may indirectly result in genotoxicity *via* the production of oxygen derived radicals that damage DNA. Despite this property of inducing oxidative stress, hydroxymethyl furanone possesses anti-oxidant properties under certain conditions as shown by Kataoka *et al.* (1997). Kataoka *et al.* (1997) also demonstrated that hydroxymethyl furanone possesses anti-tumor properties. In this study, hydroxymethyl furanone was shown to reduce the formation of benzo[a]pyrene-induced forestomach neoplasia in mice. Hydroxymethyl furanone was administered to female ICR mice (n = 28 to 30/group) with benzo[a]pyrene-induced forestomach neoplasia *via* drinking water at levels of either 0 (control), 25, 50, or 75 ppm for a period of 17 weeks (*i.e.,* from 14 to 30 weeks of age). Body weight and food intake were recorded once and 3 times weekly, respectively. At 30 weeks of age, mice were killed and tumors from forestomachs 1 mm or larger in diameter were counted using a dissecting microscope. No effect on body weight was reported in mice fed diets containing hydroxymethyl furanone compared to the control; however, a significant increase in food intake was reported in mice fed 75 ppm of hydroxymethyl furanone compared to controls. Administration of 50 and 75 ppm of hydroxymethyl furanone resulted in a significant reduction in the number of tumors per mouse and the number of tumors per tumor-bearing mouse compared to the control. Additionally, as mentioned in Section IV.B.4, JECFA reported that 3-(2*H*)-furanone derivatives were not carcinogenic in rats administered doses of up to 200 mg/kg body weight/day for 1 to 2 years (JECFA, 2006b). The lack of carcinogenic effects of 3-(2*H*)-furanone derivatives, including hydroxymethyl furanone, is accounted for by the fact that these compounds are readily excreted in the urine due to glucuronidation, a detoxification process that does not occur *in vitro*. Hydroxymethyl furanone would undergo glucuronidation at the hydroxyl moiety. Furthermore, the negative results observed in the mutagenicity study

conducted with Ester-C® containing hydroxymethyl furanone demonstrate that hydroxylmethyl furanone is not mutagenic at the levels provided by the ingredient.

2. *In vivo* Studies

In vivo mutagenicity and genotoxicity studies have not been conducted on Ester-C® and were not identified for hydroxymethyl furanone; however, one *in vivo* study conducted with calcium L-threonate was discussed in the 2008 EFSA publication. The study was a mouse bone marrow micronucleus assay, and the results demonstrated no changes in the frequency of micronucleated polychromatic erythrocytes when male mice (n=6) were orally administered calcium L-threonate at doses of up to 20,000 mg/kg body weight by gavage (Gao *et al.*, 1997d).

3. Other Studies

Carcinogenicity studies have not been conducted on Ester-C® and were not identified for calcium L-threonate; however, one study on hydroxymethyl furanone summarized in the toxicological monograph for tetrahydroxyfuran and furanone derivatives prepared by the JECFA (JECFA, 2006b) included endpoints related to carcinogenicity.

In the study by Munday and Kirkby (1973), Colworth Wistar rats (4/sex/group) were administered diets containing a meat flavor cocktail at a concentration of 197, 492, 983, 1,966, 2,949, or 3,932 ppm, providing doses of hydroxymethyl furanone of approximately 7, 18, 36, 73, 109, and 146 mg/kg body weight/day, respectively, for a period of 13 weeks, and all animals were subsequently administered diets containing the flavor cocktail at the highest dose tested (3,932 ppm, equivalent to 146 mg hydroxymethyl furanone/kg body weight/day) from Weeks 15 to 52 (*i.e.,* an additional 37 weeks). The authors concluded that the administration of diets containing the flavor cocktail at a concentration of up to 3,932 ppm (providing approximately 146 mg hydroxymethyl furanone/kg body weight/day) for a period of 1 year resulted in no effects on type, incidence, or time of development of tumors in Colworth Wistar rats.

IV.F Studies in Humans

Human studies have been conducted with two different formulations of Ester-C®, one containing both calcium threonate and hydroxymethyl furanone, and the other containing calcium threonate (but not hydroxymethyl furanone). The 2 Ester-C® formulations are essentially equivalent, differing in the specified content of hydroxymethyl furanone, which occurs in the ingredient at minimal levels. Therefore, safety data on the Ester-C® ingredient containing calcium threonate are directly relevant in the safety assessment of the Ester-C® ingredient that also specifies a content of hydroxymethyl furanone.

Ester-C®, containing both calcium threonate and hydroxymethyl furanone, was assessed for its effects on health-related endpoints in a randomized, double-blind, cross-over trial conducted in

50 healthy volunteers (11 men and 39 women) with a mean age of 44 years (Moyad *et al.*, 2009). Participants were given tablets of either Ester-C® or ascorbic acid at a dose providing 1,000 mg of vitamin C (the dose of Ester-C® was not specified) for 5 days and then at a dose providing 2,000 mg of vitamin C for the next 5 days. Therefore, each arm of the study consisted of a total of 10 consecutive days of supplementation, which was followed by a 7-day washout period. The health-related endpoint measured was 24-hour urinary oxalate levels as high ascorbic acid may potentially result in hyperoxaluria, which in turn may result in the formation of kidney stones. Adverse event monitoring also was conducted. Compared to baseline measurements, no significant differences were observed in mean total urinary oxalate excretion following either Ester-C® or ascorbic acid intake. Additionally, 6% fewer directly or indirectly related adverse events were reported with Ester-C® supplementation compared to ascorbic acid intake.

The safety and tolerance of Ester-C®, containing calcium threonate but not hydroxymethyl furanone, was assessed in a randomized, double-blind, cross-over trial in 50 healthy volunteers (26 men and 24 women) between the ages of 22 and 71 years (Gruenwald *et al.*, 2006). Subjects (25 per group) were given tablets of Ester-C® at a dose providing 1,000 mg/day of ascorbic acid (the dose of Ester-C® was not specified) or 1,000 mg/day of ascorbic acid for 3 consecutive days followed by a washout period of 3 days and a cross-over period of 3 consecutive days. A total of 3 examinations were conducted by the clinical investigator during the study period. Subjects were instructed to document the incidence and severity of epigastric symptoms once a day at the end of the day. Such symptoms included epigastric pain, heartburn, nausea, and diarrhea. In addition, general physical condition and possible occurrence of adverse events were recorded. Twenty-eight (28) subjects experienced a total of 88 epigastric symptoms that were predominantly mild in severity during the course of the study. Only 4 instances of moderate symptoms were reported, occurring equally in the Ester-C® and ascorbic acid phases, and no severe symptoms were reported. Thirty-three (37.5%) of these symptoms occurred during the Ester-C® phase, while 55 (62.5%) occurred during the ascorbic acid phase. In addition, of the 28 subjects experiencing epigastric symptoms, 4 (14.3%) experienced the symptoms only after Ester-C® intake, 15 (53.6%) experienced the symptoms only after ascorbic acid intake, and 9 (32.1%) reported epigastric symptoms after both Ester-C® and ascorbic acid intake. Tolerability to Ester-C® was rated by 72% of subjects as "very good", while that of ascorbic acid was rated as "very good" by 54% of subjects. The clinical investigator rated the tolerability of Ester-C® and ascorbic acid as "very good" for 76 and 56% of the participants, respectively. No adverse events were experienced by subjects and no subjects dropped out of the study. In addition, clinical parameters of heart rate, blood pressure, and body temperature were unaltered by Ester-C® or ascorbic acid intake compared to baseline measurements. The results of this study support the safety of and good tolerability to Ester-C®.

In an earlier double-blind study conducted on Ester-C® (containing calcium threonate but not hydroxymethyl furanone), 168 healthy volunteers (27 men and 141 women, mean age of

48 years) were randomized (84 subjects per group) to receive either two 500-mg tablets/day of Ester-C® (1,000 mg total dose) or a matched placebo with their main meal for 60 days (Van Straten and Josling, 2002). Although the study was aimed at assessing general well-being and cold symptoms with or without Ester-C® intake, study parameters also included measurements relevant to the tolerability of Ester-C®. In this regard, the authors reported that the overall incidence of side effects was low, with indigestion being the most common side effect at a rate of 4% in the Ester-C® group and 10% in the placebo group. In addition, incidences of heart burn were reported in 4% of subjects in the Ester-C® group and 7% of subjects in the placebo group. Two subjects withdrew from the study due to personal reasons. Thus, the tolerability to Ester-C® was comparable to that of the placebo.

The safe use of calcium ascorbate formulations containing calcium threonate, but not hydroxymethyl furanone, also was demonstrated in a more recent study conducted to evaluate the kinetics of ascorbic acid following an acute oral intake (Moyad *et al.*, 2008). The study consisted of a randomized, double-blind, placebo-controlled, 4-way crossover design. Fifteen (15) men, 9 non-smokers and 6 chronic smokers, aged 18 to 39 years, were given 1 tablet of 1,000 mg of ascorbic acid, calcium ascorbate at a dose providing 1,000 mg of ascorbic acid (the dose of calcium ascorbate was not specified) with a 1% calcium threonate content, calcium ascorbate with a 3% calcium threonate content, or placebo. Each intake was followed by a 7-day washout period. Although a detailed safety assessment was not conducted, the investigators reported that none of the subjects dropped out of the study and no significant adverse effects were reported by subjects during the course of the study.

Collectively, the available human data on Ester-C® and other calcium ascorbate preparations indicate that human consumption of Ester-C® at the intended use levels is not expected to be associated with any adverse effects.

IV.G Nutritional Considerations

1. Effects on Nutrient Absorption

1.1 Iron

Ascorbic acid is a known enhancer of inorganic iron (*i.e.*, iron salts) absorption and bioavailability when consumed together (Lopez and Cámara Martos, 2004; Teucher *et al.*, 2004). Ascorbic acid increases the percentage of iron absorbed in a linear fashion and does so by acting as a chelator for iron as well as a reducing agent. Consequently, ascorbic acid interacts with ferric iron [iron (III)] in the gastrointestinal tract, reducing and maintaining it in the ferrous state [iron (II)], which is the readily absorbed form of iron. Ascorbic acid also enhances iron bioavailability by releasing iron from its interaction with inhibitors, such as phytic acid and polyphenols, although an increase in the ascorbic acid to iron ratio is required (Teucher *et al.*, 2004). Therefore, the ingestion of iron-fortified foods with foods fortified with sources of

ascorbic acid, such as Ester-C®, may increase the bioavailability of iron; however, this is considered to be favorable in order to counteract inhibitors of iron absorption present in many foods. The enhancing effect of ascorbic acid on iron bioavailability from fortified foods is not of safety concern as the level of iron used to fortify foods is limited to a level that would aid in achieving, and not exceeding, the RDA for iron.

1.2 Copper

In animals, ascorbic acid acts as an inhibitor of copper absorption and bioavailability. This is largely due to the ascorbic acid-mediated reduction of cupric copper [copper (II)] to cuprous copper [copper (I)] in the intestine (Lonnerdal, 1996; Wapnir, 1998). Cuprous copper is the poorly absorbed form of copper. In humans, the effect of high ascorbic acid intake on the intestinal absorption of copper is much less pronounced with no effects observed at doses of up to 605 mg ascorbic acid/day (Lonnerdal, 1996; Wapnir, 1998). Therefore, foods containing sources of ascorbic acid, such as Ester-C®, at equivalent levels are not expected to have an appreciable effect on copper absorption.

IV.H Expert Panel Evaluation

The Ester C Company has determined that Ester-C® [calcium ascorbate with threonate] is GRAS for use as a nutrient in traditional foods and medical foods as described in Table I.D-1, on the basis of scientific procedures. This GRAS determination is based on data generally available in the public domain pertaining to the safety of Ester-C®, as discussed herein, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of the Ester-C® ingredient as a component of food. The Expert Panel consisted of the following qualified scientific experts: Prof. William J. Waddell, M.D. (University of Louisville School of Medicine), Prof. Gary M. Williams, M.D. (New York Medical College), and Dr. Ian C. Munro, Ph.D. (Cantox Health Sciences International).

The Expert Panel, convened by the Ester C Company, independently and critically evaluated all data and information presented herein, and also concluded that Ester-C® was GRAS for use as a nutrient in traditional food and beverage products and medical foods as described in Table I.D-1., based on scientific procedures. A summary of data and information reviewed by the Expert Panel, and evaluation of such data as it pertains to the proposed GRAS uses of Ester C's GRAS is presented in Appendix A.

IV.I Conclusion

Based on the above data and information presented herein, the Ester C Company has concluded that the intended food uses of Ester-C, as described in Table I.D-1, are GRAS based on scientific procedures. General recognition of The Ester C Company's GRAS determination is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified

by experience and scientific training, to evaluate the use of Ester-C® in food, who similarly concluded that the intended uses of Ester-C® described herein are GRAS.

Ester-C® therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR.

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APPENDIX A

APPENDIX A

EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF ESTER-C® CALCIUM ASCORBATE FOR USE IN FOODS

EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF ESTER-C® CALCIUM ASCORBATE FOR USE IN FOODS

August 3, 2011

INTRODUCTION

At the request of the Ester C Company, an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether, under the conditions of intended use as a food ingredient, Ester-C® Calcium Ascorbate (Ester-C®), would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Panel consisted of the below-signed qualified scientific experts: Prof. William J. Waddell, M.D. (University of Louisville School of Medicine), Prof. Gary M. Williams, M.D. (New York Medical College), and Dr. Ian C. Munro, Ph.D. (Cantox Health Sciences International).

The Panel, independently and collectively, critically examined a comprehensive package of scientific information and data compiled from the literature and other published sources through May of 2009. In addition, the Panel evaluated other information deemed appropriate or necessary, including data and information provided by the Ester C Company. The information evaluated by the Panel included details pertaining to the method of manufacture and product specifications, supporting analytical data, intended use-levels in specified foods, consumption estimates for all intended uses, and a comprehensive assessment of the available scientific literature pertaining to the safety of Ester-C® and its defining components.

Following independent, critical evaluation of such data and information, the Panel convened on 8 May 2009 and unanimously concluded that the intended uses in traditional foods described herein for Ester-C®, meeting appropriate food-grade specifications as described in the supporting dossier [Documentation Supporting the Generally Recognized as Safe (GRAS) Status of Ester-C® Calcium Ascorbate for Use In Traditional Food Products] and manufactured consistent with current Good Manufacturing Practices (cGMP), are GRAS based on scientific procedures[1]. A summary of the basis for the Panel's conclusion is provided below.

[1] Additional data pertaining to the stability of Ester-C® were generated following the Panel meeting in May 2009; however, Dr. Munro was unable to review the data and sign the Expert Panel Consensus Statement prior to his passing. Dr. Ashley Roberts, Ph.D. (Cantox Health Sciences International) subsequently evaluated the data and information and concluded that the intended uses of Ester-C® are GRAS based on scientific procedures.

SUMMARY AND BASIS FOR THE GRAS STATUS OF ESTER-C® CALCIUM ASCORBATE

The Ester C Company's Ester-C® ingredient consists of calcium ascorbate and calcium threonate, and comprises 76.60% ascorbic acid, 9.18% calcium, and 1.15% threonic acid. Ester-C® also contains minimal levels of 4-hydroxy-5-methyl-3(2*H*)-furanone (hydroxymethyl furanone) (0.045%), a reaction by-product. All constituents of Ester-C® occur naturally in many foods, and therefore, are commonly consumed as part of the normal human diet and have well-established long histories of safe consumption. Ascorbic acid is an essential vitamin that occurs predominantly in fruits and vegetables, and calcium is an essential mineral that occurs predominantly in dairy products. L-Threonic acid is a known oxidative degradation product of ascorbic acid (Tatum *et al.*, 1969; Deustch, 1998; Knafo *et al.*, 2005; Debolt *et al.*, 2007) and occurs naturally in foods that contain ascorbic acid. Threonic acid also has been detected in bread (Thewlis, 1971) and in drinking water (Jansson *et al.*, 2004). Hydroxymethyl furanone is an ascorbic acid-related compound that occurs naturally in cooked foods as a result of the Maillard reaction that takes place between pentose sugars and amino acids during cooking (Slaughter, 1999). Such foods include, but are not limited to, beef broth, roasted sesame seeds, bread crust, soy sauce, malt, beer, popcorn, and cooked clam (Slaughter, 1999). In addition, hydroxymethyl furanone is present in the aroma of blackberries (Klesk and Qian, 2003) and occurs naturally in guava, raspberries, and tomatoes (Buttery *et al.*, 1994; Burdock, 2009).

Ester-C® is produced in accordance with cGMP using food-grade quality reagents and meets appropriate food-grade specifications. The raw materials include ascorbic acid (21 CFR §182.3013 and 21 CFR §182.8013) (U.S. FDA, 2011), calcium carbonate (21 CFR §184.1191) (U.S. FDA, 2011), and calcium L-threonate. Calcium L-threonate has not been evaluated for its GRAS status. Therefore, the use of L-threonic acid, as calcium L-threonate, in the manufacture of Ester-C® also was subject to the GRAS evaluation herein.

Ester-C® is produced in bulk with precise temperature control using a water-based method. During this process, calcium ascorbate is produced as a result of a reaction between ascorbic acid and calcium carbonate under steam pressure. Calcium L-threonate (*i.e.*, the calcium salt of L-threonic acid) also is added intermittently at the start of the manufacturing process and occurs in the final ingredient. Hydroxymethyl furanone also occurs in the final ingredient as a by-product of the reaction. A purification step is not included and no processing aids are used. As per the final product specifications, Ester-C® is composed of 76.60 mg/100 mg ascorbic acid, 9.18 mg/100 mg calcium, 1.15 mg/100 mg threonic acid, and 0.045 mg/100 mg hydroxymethylfuranone, and analyses of 5 non-consecutive lots demonstrate a product consistent with the specifications. In addition, analysis of the Ester-C® ingredient for lead and microbes indicate conformance to the limits set by the product specifications. Furthermore, Ester-C® is free of genetically modified organisms, contains no materials of animal origin, is free from artificial colors, flavors, and preservatives, is not irradiated or sterilized, and does not contain any carriers. The stability of the bulk ingredient and conformance to the product

specifications was demonstrated under room temperature and accelerated conditions. Based on the results of these studies, a 24-month (2-year) shelf life was established for the bulk ingredient. The stability of Ester-C® in, powdered drink mixes, cereal based bars, orange juice, and cranberry juice concentrate also was demonstrated under the intended storage conditions.

Ester-C® is intended for use in foods and beverages, including beverages and beverage bases, breakfast cereals, chewing gum, coffee and tea, grain products, and processed fruits and fruit juices. In those foods and beverages already fortified with a source of vitamin C, Ester-C® is intended to be added in replacement of the existing vitamin C fortificant, and not in addition to. Ester-C® is intended for use in foods and beverages at use levels of 0.033 to 0.52%, providing 60 to 250 mg vitamin C per serving, depending on the food or beverage. In chewing gum, the intended use level of Ester-C® is 5.44%, providing 125 mg of vitamin C per serving. Ester-C® also is intended for use in medical foods at use levels providing up to 500 mg of vitamin C per serving.

The consumption of Ester-C® from all intended food uses was estimated using the National Center for Health Statistics' (NCHS) National Health and Nutrition Examination Surveys (NHANES) for the years 2003-2004 (NHANES 2003-2004) (CDC, 2006; USDA, 2009), which provide the most appropriate data for evaluating food use and food consumption patterns in the United States (U.S.). The estimated intakes for the total U.S. population are summarized in Table 1 on an all-user and per person basis along with the reported background dietary intakes of Ester-C® constituents.

Table 1 Summary of the Estimated Daily Intake of Ester-C® and of the Individual Constituent from All Intended Food Categories in the United States by the Total Population on a All-User Basis (2003-2004 NHANES Data)

Ester-C® and its individual constituents	Background Dietary Intake by Total U.S. Population[a]		All-Users Consumption of Ester-C® by Total U.S. Population	
	Mean (mg)	90th Percentile (mg)	Mean (mg)	90th Percentile (mg)
Ester-C®	N/A	N/A	373	810
Vitamin C (ascorbic acid)	106 (90 to 140)	150 (120 to 210)	286	620
Calcium	797 (460 to 1,170)	1,241 (670 to 2,040)	34	74
Threonic acid	N/A	N/A	4	9
Hydroxymethyl furanone	0.00007[b] and 0.84[c]	N/A	0.168	0.364

Abbreviations: N/A = not applicable
[a] Numbers in parentheses represent the range of intakes across all population groups.
[b] As reported by JECFA (2006).
[c] As reported by FEMA.

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Under the conditions of intended use of Ester-C® in food, total U.S. population all-user mean and 90th percentile daily intakes from all proposed food uses were estimated to be 373 mg/person/day (8 mg/kg body weight/day) and 810 mg/person/day (18 mg/kg body weight/day), respectively. The estimated all-user mean daily intake of Ester-C® is equivalent to intakes of 286 mg ascorbic acid/person/day (6 mg/kg body weight/day), 34 mg calcium/person/day (0.73 mg/kg body weight/day), 4 mg threonic acid/person/day (92 µg/kg body weight/day), and 168 µg hydroxymethyl furanone/person/day (4 µg/kg body weight/day) by the total U.S. population. At the estimated all-user 90th percentile daily intake of Ester-C®, intakes of 620 mg ascorbic acid/person/day (14 mg/kg body weight/day), 74 mg calcium/person/day (1.66 mg/kg body weight/day), 4 mg threonic acid/person/day (208 µg/kg body weight/day), and 364 µg hydroxymethyl furanone/person/day (8 µg/kg body weight/day) were calculated for the total U.S. population. In comparison, estimated mean background dietary intakes of vitamin C and calcium in the U.S. are in the range of 90 to 140 mg/person/day (IOM, 2000, Appendix C) and 460 to 1,170 mg/person/day (IOM, 1997, Appendix D), respectively. At the 90th percentile, background dietary intakes of vitamin C are in the range of 120 to 210 mg/person/day (IOM, 2000, Appendix C) and those of calcium are in the range of 670 to 2,040 mg/person/day (IOM, 1997, Appendix D). For hydroxymethyl furanone, the Joint FAO/WHO Expert Committee of Food Additives (JECFA) has reported daily intakes of hydroxymethyl furanone of 0.056 and 0.00007 mg/person/day for the European and U.S. populations, respectively (JECFA, 2006), and the U.S. Flavor and Extract Manufacturers' Association (FEMA) reported a Possible Average Daily Intake (PADI) of 0.84 mg by the U.S. population. The background dietary intake of calcium threonate or threonic acid was not identified in the literature.

In addition, the estimated intakes of vitamin C are above the Recommended Daily Allowance (RDA) of 90 mg/day for adult men and 75 mg/day for adult women established by the Food and Nutrition Board of the Institute of Medicine (IOM, 2000). Therefore, background dietary intake alone appears to be sufficient in achieving the RDA. Such background dietary intakes of vitamin C, however, include food and beverage sources to which vitamin C is added. As Ester-C® is intended, in part, to replace existing vitamin C fortificants in many foods and beverages, the intended use of Ester-C® will, in this respect, aid in achieving the RDA for vitamin C established by the IOM (2000) (90 mg/day for adult men and 75 mg/day for adult women).

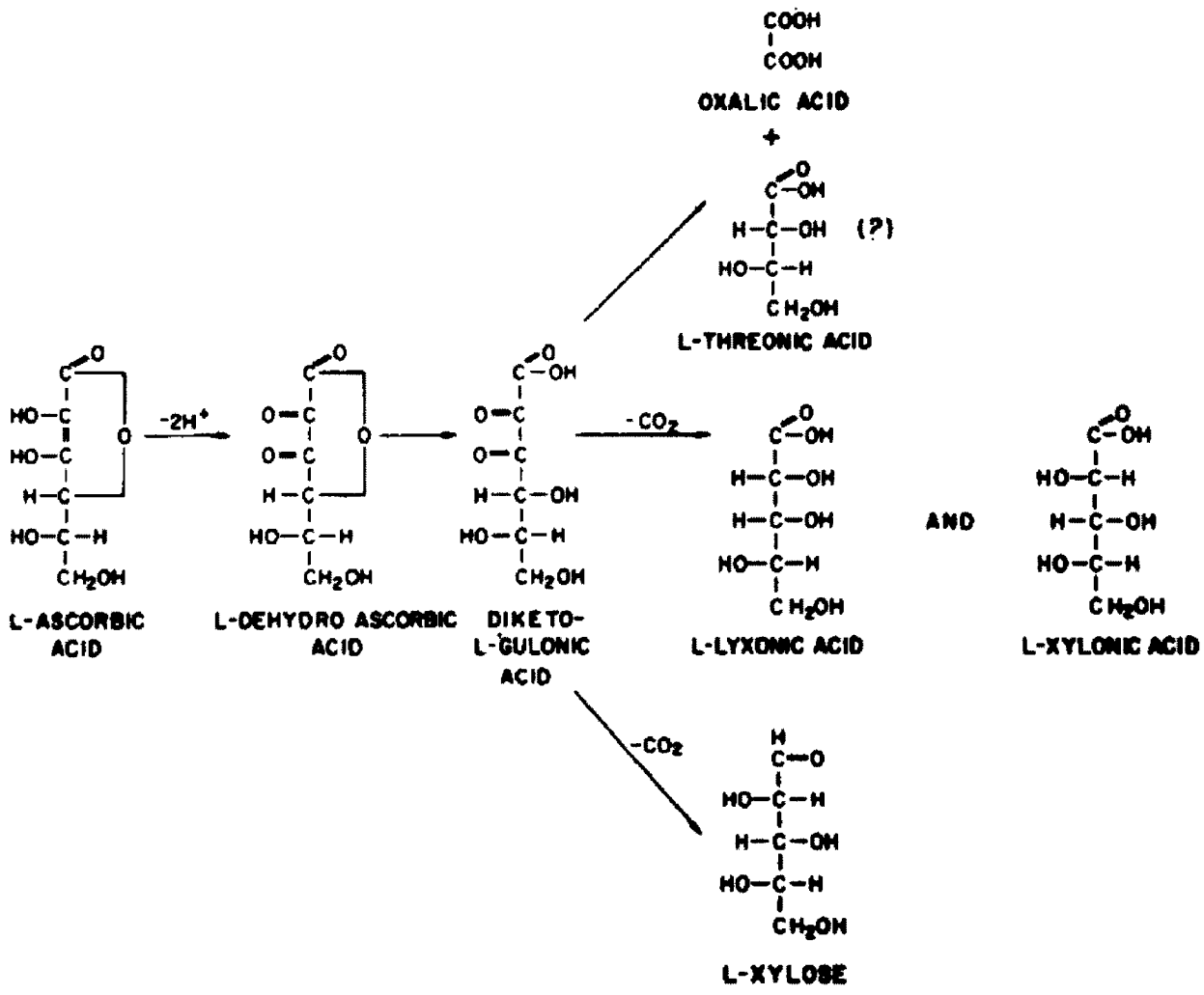
Based on the intakes analysis, the intended use of Ester-C® may increase the total daily intakes of vitamin C as it is intended for use in foods to which vitamin C is not currently added; however, as Ester-C® is intended, in part, to replace existing vitamin C fortificants in many foods and beverages, the increase in vitamin C intakes will not be entirely additive. Although the anticipated vitamin C intake from the intended conditions of use of Ester-C® are above current background intake levels, it is expected not to be of concern to human health considering that these intakes of vitamin C from Ester-C® are cumulative of smaller intakes taken with food

throughout the day as opposed to a bolus intake, and remain below the Tolerable Upper Intake Levels (UL) of 2,000 mg/person/day for adults set by the IOM (IOM, 2000).

The fate of Ester-C® constituents, ascorbic acid, calcium, threonic acid, and hyroxymethyl furanone, following ingestion was considered as part of the toxicological data package. Following ingestion, calcium ascorbate is hydrolyzed in the stomach and dissociates into its constituent ions, calcium, and ascorbate. Gastric acid (hydrochloric acid) in the stomach then converts the ascorbate ions into ascorbic acid. Calcium and ascorbic acid are subsequently absorbed from the small intestine into the systemic circulation. This is supported by observations in both humans (Johnston and Luo, 1994; Moyad *et al.*, 2008; Pancorbo *et al.*, 2008) and experimental animals (Bush and Verlangieri, 1987; Tsugawa *et al.*, 1999; Wang *et al.*, 2001; Cai *et al.*, 2004) demonstrating a rapid rise in plasma ascorbic acid and calcium concentrations following a single oral dose of various calcium ascorbate formulations, including Ester-C® (containing calcium threonate, but not hydroxymethylfuranone). The absorption and pharmacokinetic profile of ascorbic acid from Ester-C® is similar to that documented for ascorbic acid itself (Blanchard *et al.*, 1990; Johnston and Luo, 1994; Bluck *et al.*, 1996; Graumlich *et al.*, 1997; Bates *et al.*, 2004; Padayatty *et al*, 2004; Moyad *et al.*, 2008; Pancorbo *et al.*, 2008).

At typical dietary intakes of ascorbic acid (30 to 180 mg/person/day), absorption of the vitamin is efficient (Kallner *et al.*, 1979) and occurs *via* a sodium-dependent active transport mechanism, which is dose-dependent and saturable at higher intake levels (Kallner *et al.*, 1979; Blanchard *et al.*, 1997; Graumlich *et al.*, 1997; Rumsey and Levine, 1998; Padayatty *et al*, 2004). Ascorbic acid is widely distributed throughout the human body, with the highest concentrations reported in the adrenal and pituitary glands, and lower levels observed in the liver, spleen, lungs, kidneys, testes, thyroid, heart, skeletal muscle, brain, pancreas, and eyes (Hornig, 1975). Distribution of ascorbic acid to tissues also is a saturable process, with intracellular stores becoming saturated at doses of 200 mg/person and greater (Graumlich *et al.*, 1997). Ascorbic acid is metabolized *via* oxidation to dehydroascorbic acid, which can be reduced back to ascorbic acid or further oxidized to diketogulonic acid, which, in turn, is oxidized to oxalic acid, threonic acid, xylose, xylonic acid, and lyxonic acid (Figure 1) (Burns, 1975). Hydroxymethyl furanone may also be a biological metabolite of ascorbic acid in humans (Moyad *et al.*, 2009). In addition, ascorbic acid may undergo conjugation with sulfate to form ascorbate-2-sulfate (Baker *et al.*, 1975; Tolbert, 1985). The bulk of an absorbed dose of ascorbic acid from Ester-C®, calcium ascorbate, or ascorbic acid is eliminated rapidly from the circulation (Bush and Verlangieri, 1987; Blanchard *et al.*, 1990; Graumlich *et al.*, 1997; Wang *et al.*, 2001; Moyad *et al.*, 2008; Pancorbo *et al.*, 2008) and reabsorbed by the kidneys or excreted in the urine at higher intake levels along with any metabolites (Kallner *et al.*, 1979; Blanchard *et al.*, 1990, 1997).

Figure 1 Metabolism of ascorbic acid to urinary metabolites (from Burns, 1975)

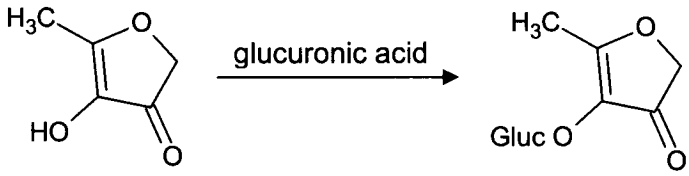


Calcium also is absorbed from the small intestine *via* a saturable active transport mechanism, which is vitamin D-dependent, at relatively low dietary intakes (Bronner, 2003). Calcium then enters the circulation following intracellular diffusion and extrusion from enterocytes *via* an adenosine triphosphatase pump. When dietary calcium intakes are high, however, calcium is absorbed paracellularly by diffusion through tight junctions (Bronner, 2003). The predominant localization of calcium within the body is in mineralized tissues, such as bone and teeth, where it is present as calcium phosphate (IOM, 1997). Ninety-nine percent (99%) of the body's calcium stores are found in these tissues and the remaining 1% occurs in blood, extracellular fluid, muscle, and other tissues (IOM, 1997). Calcium is excreted from the body by both urinary and fecal routes (Cashman, 2002; Cai *et al.*, 2004; Bhatia, 2008).

Whether threonic acid is absorbed following ingestion of threonic acid itself is unknown; however, threonic acid occurs in the human body as a metabolite of ascorbic acid (Burns, 1975; Deutsch *et al.*, 1999; Harding *et al.*, 1999). Threonic acid is excreted unchanged in urine in both animals and humans (Knafo *et al.*, 2005; Wang *et al.*, 2006). No other information on the metabolic fate of threonic acid *in vivo* was available for review.

Furanone derivatives that are structurally similar to hydroxymethyl furanone are documented as readily absorbed and rapidly eliminated in urine (Roscher *et al.*, 1997; Hiramoto *et al.*, 1998). The furanone DMHF has been reported as being is excreted in urine as a glucuronide conjugate in humans (Roscher *et al.*, 1997). Neither free DMHF nor other DMHF conjugated metabolites were detected in urine. Given the structural similarity to DMHF, hydroxymethyl furanone also is expected to be rapidly absorbed from the gastrointestinal tract, metabolized *via* conjugation with glucuronic acid, and subsequently excreted in the urine (Figure 2).

Figure 2 Metabolic fate of hydroxymethyl furanone



urinary metabolite

Studies evaluating the subchronic toxicity and mutagenicity of Ester-C®, as well as safety and tolerance in humans were used to evaluate the safety of Ester-C® for human consumption. Safety data on of Ester-C®, containing calcium threonate but not hydroxymethyl furanone, including one acute toxicity study, one subchronic toxicity study, and a human study, also were reviewed to assess the potential toxicity of Ester-C®. The two Ester-C® formulations are essentially equivalent, differing in the specified content of hydroxymethyl furanone, which occurs in the ingredient at minimal levels. Therefore, safety data on the Ester-C® ingredient containing calcium threonate are directly relevant in the safety assessment of the Ester-C® ingredient that also specifies a content of hydroxymethyl furanone. Since calcium L-threonate, used in the manufacture of Ester-C®, also is subject to GRAS evaluation herein, and since it occurs in the final ingredient, toxicity data on calcium threonate and threonic acid were subject to review. The latter data included studies assessing acute and subchronic oral toxicity, developmental and reproductive toxicity, and mutagenicity and genotoxicity. To further evaluate the safety of Ester-C®, toxicity data on hydroxymethyl furanone, including subchronic oral toxicity studies and mutagenicity and genotoxicity studies were reviewed.

The results of animal toxicity studies conducted on Ester-C®, as well as studies on calcium L-threonate and hydroxymethyl furanone are supportive of the safety of Ester-C® for human

consumption at the intended levels of use. A single oral dose of 7,500 mg/kg body weight of the Ester-C® ingredient with a content of calcium threonate but no hydroxymethyl furanone did not result in acute toxicity in rats (EFSA, 2007). Similarly, neither calcium threonate nor the hemicalcium salt of L-threonic acid induced signs of toxicity when orally administered to rats at a dose of 5,000 mg/kg body weight (EFSA, 2007). Calcium L-threonate was further not acutely toxic to Kunming mice or Wistar rats when orally administered at a dose of 40,000 and 32,000 mg/kg body weight, respectively (Gao *et al.*, 1997a).

The 28-day subchronic study conducted on Ester-C® indicated that repeated oral administration of Ester-C® at dietary levels of up to 100,000 ppm (10%) was well-tolerated and not associated with any compound-related adverse effects or death in Hsd:SD® rats (Eurofins Product Safety Laboratories, 2007). The study was conducted in compliance with the Organisation for Economic Co-Operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (OECD, 1998), with the exception that the serology analysis was not conducted under GLP compliance. The study also was conducted in accordance with the FDA and OECD guidelines for the toxicological testing of chemicals (U.S. FDA, 2003; OECD, 2008). All animals survived the course of the study. Ophthalmoscopic examination of both eyes of all animals was unremarkable. Although transient soft feces were observed at the 50,000 ppm (males and females) and 100,000 ppm (males only) levels, this effect was considered as non-adverse due to recovery shortly thereafter, and was likely the result of the osmotic effect of unabsorbed ascorbic acid passing through the gastrointestinal tract. Incidental reductions in body weight gain, food consumption, and food efficiency also were observed in both sexes; however, these changes were considered toxicologically insignificant as they were transient and occurred at different intervals of time among the two sexes. Such changes also were non-adverse in nature. Compared to controls, clinical chemistry, urinalysis, and hematology results revealed a number of statistically significant sporadic changes; however these changes were deemed incidental and toxicological insignificant as they were non-dose-dependent and/or were observed in one sex only. Changes in organ weights also were sporadic and not accompanied by relevant clinical or histopathological findings, and thus, were considered toxicologically insignificant. Macroscopic examination did not reveal any abnormalities and no compound-related microscopic changes were observed. Based on the results and under the conditions of this study, the authors concluded the no-observed-adverse-effect level (NOAEL) for Ester-C® to be 100,000 ppm or 10% in the diet for male and female rats, which is equivalent to 8,331 and 8,744 mg/kg body weighty/day, the highest dose tested. One other study on Ester-C® was conducted, which investigated the effect of repeat oral administration of the ingredient (containing calcium threonate but not hydroxymethyl furanone) in improving the scorbutic condition of experimental rats (Verlangieri *et al.*, 1991). Although, the safety of Ester-C® was not specifically addressed, the results of this study demonstrated that oral administration of Ester-C® at a level of 0.08 mg/mL in drinking water was not overtly toxic to mutant Wistar rats during the course of the 24-day study. In this respect, animals displayed normal continuous body weight gain throughout the study period and no signs of morbidity were

observed. Additionally, rats provided with Ester-C® displayed very low scores for scurvy-induced symptoms compared to an ascorbic acid control group. Together, the results of the animal studies conducted with Ester-C® demonstrate that repeated oral administration of high doses of Ester-C® does not produce adverse effects in rats at dietary intakes of up to approximately 8,000 mg/kg body weight/day, and support the safety of Ester-C® under the intended conditions of use.

With regards to short-term repeated oral administration of threonic acid, no significant differences in food intake, body weights/growth rate, select clinical chemistry and hematology parameters, or most organ weights were observed in male albino Dunkin-Hartley guinea pigs administered threonic acid at a dose of 100 mg/kg body weight/day for 28 days (Thomas and Hughes, 1983) or in male albino Wistar rats administered 1,000 mg/kg body weight/day of calcium threonate for 120 days (Thomas and Hughes, 1985). Additionally in guinea pigs, plasma levels of indicators of liver toxicity were similar between threonic acid and control groups. In rats, a significant, yet slight, decrease in relative liver weight observed in calcium threonate-fed rats compared to control rats; however, based on the overall results of the study, the authors concluded that there was "an essential lack of toxicity of threonic acid in rats and mice at dietary concentrations very much in excess of any amounts likely to be ingested by humankind". Although the lifespan of scorbutic guinea pigs receiving long-term supplemental threonic acid was significantly, yet marginally, reduced compared to control animals, this effect was likely due to ascorbic acid depletion in these animals as threonic acid itself had no effect on a number of physiological and biochemical characteristics customarily regarded as being of significance in toxicity studies. In support of this, no significant difference was observed in the life span of mice fed diets containing up to 0.20% calcium threonate (300 mg/kg body weight /day) compared to control mice, and no deaths occurred following calcium L-threonate administration to rats and dogs as described in the 24-week studies summarized below.

Two 24-week oral subchronic toxicity studies were conducted on calcium L-threonate in rats and dogs (Zhao *et al.*, 1997; Gao *et al.*, 1998), which were reviewed and summarized by the European Food Safety Authority's (EFSA) Scientific Panel on Food Additives and Nutrient Sources in their opinion on the use of calcium L-threonate as a source of calcium in food supplements (EFSA, 2008). In these studies, no deaths occurred following oral administration of calcium L-threonate at doses of up to 3,000 and 6,000 mg/kg body weight/day in hybrid dogs and rats (strain not specified), respectively. Several significant effects were observed in male and female rats belonging to the high-dose group (6,000 mg/kg body weight/day) compared to controls. These changes included incidences of decreased spontaneous motor activity and loose stools, reduced body weight, shorter coagulation time, presence of gas and yellow liquid in the intestines, and mild thyroid gland accretion (males only). Likewise, slight hyperplasia of the thyroid gland accompanied by histopathological findings was reported to occur in dogs following administration of calcium L-threonate at doses of 3,000 and 6,000 mg/kg body weight/day. The effects observed on coagulation time and in the thyroid gland were suggested

by the authors to be a result of high calcium intake. No other gross or histopathological findings were reported to occur at any dose in either rats or dogs. Additionally in rats, no changes in clinical appearance, clinical chemistry, and hematology and were reported to occur in animals administered 2,000 or 4,000 mg/kg body weight/day of calcium L-threonate. In dogs, no differences in general appearance, psychomotility, appetite, feed and water intake, urinalysis, clinical chemistry, and hematology were reported to occur in any of the calcium L-threonate groups compared to the control group. Based on the effects observed in the high-dose groups, the Scientific Panel determined the NOAEL for calcium L-threonate to be 4,000 mg/kg body weight/day in rats. In dogs, the NOAEL for calcium L-threonate was determined to be 1,000 mg/kg body weight/day in dogs based on the effects observed in the mid- and high-dose groups. Therefore, the low levels of calcium threonate provided by Ester-C® are not anticipated to result in adverse effects in humans.

The potential subchronic toxicity of hydroxymethyl furanone was assessed in 4 studies conducted in rats (strain not specified), which were reviewed and summarized by JECFA in the Committee's toxicological monograph for tetrahydroxyfuran and furanone derivatives, including hydroxymethyl furanone (JECFA, 2006). In these studies, hydroxymethyl furanone, at a level of 74.1%, was provided as a component of a meat flavor cocktail administered in the diet. Feeding of diets containing the meat flavor cocktail at concentrations of up to 3,932 ppm, providing 146 mg/kg body weight/day of hydroxymethyl furanone, for 4, 6, or 13 weeks was not associated with any compound-related adverse effects. Specifically, no compound-related adverse effects in body weight, food and water intake, food utilization, clinical chemistry, urinalysis, hematology, and organ weights were reported compared to the control. A few sporadic changes were noted in hematology in the 4- and 6- week studies; however, these were not dose-dependent, and therefore, not compound-related or toxicologically significant. Significant changes also were observed in relative liver and kidney weights in the 6- and 13-week studies, respectively; however, macroscopic examinations and histopathology did not reveal any compound-related abnormalities in any tissue. Likewise, administration of the meat flavor cocktail for 52 weeks at a dietary concentration of 3,932 ppm was not associated with any compound-related adverse effects on general health, survival, body weights, organ weights, hematology, or gross pathology in Colworth Wistar rats. The authors further concluded that the administration of diets containing the flavor cocktail at a concentration of up to 3,932 ppm (providing 146 mg hydroxymethyl furanone/kg body weight/day) for a period of 1 year resulted in no effects on type, incidence, or time of development of tumors in Colworth Wistar rats. The dietary levels of hydroxymethyl furanone tested in these studies are far in excess of those provided by Ester-C®, and therefore, the hydroxymethyl furanone content provided by Ester-C® is not anticipated to result in adverse effects in humans.

Studies examining the potential developmental and reproductive toxicity of Ester-C® have not been performed. Developmental and reproductive studies on hydroxymethyl furanone also were not identified in the literature; however, reproductive and developmental toxicity studies

have been conducted on calcium L-threonate. Details of these studies were made available *via* the EFSA's publication on the Scientific Panel on Food Additives and Nutrient Sources' opinion on the use of calcium L-threonate in food supplements (EFSA, 2008). A reproductive toxicity study conducted in Kunming mice demonstrated that administration of calcium L-threonate prior to mating during gestation at doses of 6,000 mg/kg body weight/day by gavage did not affect reproductive performance or fertility (Wu *et al.*, 1997a). *In utero* exposure to calcium L-threonate at a maternal dose of 6,000 mg/kg body weight/day from GD 0 or 6 to GD 15 also was reported to not result in embryotoxicity, fetotoxicity, or teratogenicity in Kunming mice in 2 teratology studies (Wu *et al.*, 1997b,c). Furthermore, calcium L-threonate at this level was not maternally toxic in either study. Calcium L-threonate was further investigated for its effect on maturation and postnatal development in Kunming mice. *In utero* exposure from GD 15 and onwards in combination with lactational exposure to calcium L-threonate at a maternal dose of 6,000 mg/kg body weight/day had no effect on developmental or behavior parameters compared to control. The reproductive performance and fertility of the offspring (F_1 generation) also was unaffected. Accordingly, human consumption of calcium L-threonate at the intended use levels of Ester-C® is not expected to be associated with any adverse reproductive or developmental effects.

Ester-C® did not exhibit any mutagenic potential *in vitro* in standard *Salmonella typhimurium* and *Escherichia coli* bacterial strains at concentrations of up to 5,000 µg/plate in either the presence or absence of metabolic activation (BSL Bioservice Scientific Laboratories GmbH, 2007). Therefore, Ester-C® intake at the levels provided in foods will not be genotoxic.

Calcium L-threonate also has been reported to be non-mutagenic in the bacterial reverse mutation assay using *S. typhimurium* strains at concentrations of up to 2,500 µg/mL with or without metabolic activation (Gao *et al.*, 1997b). Additionally, calcium L-threonate was reported to be non-clastogenic *in vitro* in Chinese hamster cells at concentrations of up to 5,000 µg/mL (Gao *et al.*, 1997c), and also did not increase micronucleus frequency in mouse bone marrow cells *in vivo* at oral doses of up to 20,000 mg/kg body weight (Gao *et al.*, 1997d). Furthermore, the negative results observed in the mutagenicity study conducted with Ester-C® containing both calcium threonate and hydroxymethyl furanone demonstrate that calcium threonate will not be mutagenic under the intended conditions of use of Ester-C®.

Hydroxymethyl furanone has been shown to induce a dose-dependent mutagenic response in the bacterial reverse mutation assay in *S. typhimurium* strain TA100, a strain sensitive to oxidative mutagenicity, at concentrations of up to 5,000 µg/plate with metabolic activation (Hiramoto *et al.*, 1996). A similar response was obtained in the absence of metabolic activation, except that mutagenicity was not observed at the highest concentration tested (5,000 µg/plate). Mutagenic activity was not observed in strain TA98 with or without metabolic activation. Hydroxymethyl furanone also was demonstrated to induce single strand breaks in supercoiled plasmid DNA *in vitro* in a dose- and time-dependent manner (90 to 900 µM), but only under

neutral pH conditions and not under acidic conditions. The investigators ascribed the observed effects to oxygen derived radicals. Contrary to the findings of Hiramoto *et al.* (1996), Kataoka *et al.* (1997) demonstrated that hydroxymethyl furanone is a scavenger of H_2O_2, a reactive oxygen species, at concentrations of 50 µM or greater in human PMN leukocytes. These investigators also demonstrated that hydroxymethyl furanone, at concentrations of 10^{-4} and $5x10^{-5}$ M *in vitro*, possessed lipid peroxidation inhibiting properties. Although harboring some mutagenic activity *in vitro*, hydroxylmethyl furanone failed to induce any effect on type, incidence, or time of tumor development in Colworth Wistar rats when provided a dose of 146 mg/kg body weight/day in the diet as a component of a meat flavor cocktail for 1 year. Hydroxymethyl furanone also was shown to reduce the formation of benzo[a]pyrene-induced forestomach neoplasia in mice when provided at levels of 50 and 75 ppm in drinking water over a 17-week period (Kataoka *et al.*, 1997). Additionally, JECFA reported that 3-(2*H*)-furanone derivatives were not carcinogenic in rats administered doses up to 200 mg/kg body weight/day for 1 to 2 years (JECFA, 2006). The lack of carcinogenic effects of 3-(2*H*)-furanone derivatives, including hydroxymethyl furanone, is accounted for by the fact that these compounds are readily excreted in the urine due to glucuronidation, a detoxification process that does not occur *in vitro*. Hydroxymethyl furanone would undergo glucuronidation at the hydroxyl moiety. Therefore, hydroxymethyl furanone is not expected to be genotoxic *in vivo*. Furthermore, the negative results observed in the mutagenicity study conducted with Ester-C® containing hydroxymethyl furanone demonstrate that hydroxylmethyl furanone will not be genotoxic under the intended conditions of use of Ester-C®.

Ester-C®, containing both calcium threonate and hydroxymethyl furanone, was assessed for its effects on health-related endpoints in a randomized, double-blind, cross-over trial conducted in healthy volunteers (Moyad *et al.*, 2009). Participants were given tablets of either Ester-C® or ascorbic acid at a dose providing 1,000 mg of vitamin C for 5 days and then at a dose providing 2,000 mg of vitamin C for the next 5 days. Measurement of 24-hour urinary oxalate levels revealed no significant differences in mean total urinary oxalate excretion following either Ester-C® or ascorbic acid intake compared to baseline. Additionally, 6% fewer directly or indirectly related adverse events were reported with Ester-C® supplementation compared to ascorbic acid intake.

Short-term safety and tolerance of Ester-C®, containing calcium threonate but not hydroxymethyl furanone, was assessed in 2 randomized, double-blind studies in healthy volunteers. In a cross-over study, participants were given tablets of either Ester-C® at a dose providing 1,000 mg/day of ascorbic acid or 1,000 mg/day of ascorbic acid for 3 consecutive days (Gruenwald *et al.*, 2006). Epigastric symptoms, including epigastric pain, heartburn, nausea, and diarrhea, occurred less frequently during the Ester-C® phase (33) compared to the ascorbic acid phase (55). Specifically, 28 out of the 50 subjects experienced epigastric symptoms; 4 (14.3%) experienced the symptoms only after Ester-C® intake, 15 (53.6%) experienced the symptoms only after ascorbic acid intake, and 9 (32.1%) reported epigastric

symptoms after both Ester-C® and ascorbic acid intake. Most symptoms were mild in severity and only a few moderate symptoms reported. Based on a rating scale, Ester-C® was deemed to have good tolerability. In addition, no adverse events were experienced by subjects, and heart rate, blood pressure, and body temperature were unaltered by Ester-C® or ascorbic acid intake. In another study, participants were received either two 500-mg tablets/day of Ester-C® (1,000 mg total dose) or a matched placebo with their main meal for 60 days (Van Straten and Josling, 2002). The overall incidence of side effects was observed to be low, with indigestion being the most common side effect at a rate of 4% in the Ester-C® group and 10% in the placebo group. In addition, incidences of heart burn were reported in 4% of subjects in the Ester-C® group and 7% of subjects in the placebo group. The results of the human studies conducted on Ester-C® support good tolerability to and general safety of Ester-C® at doses of up to 2,000 mg/person/day.

The safe use of calcium ascorbate formulations containing calcium threonate, but not hydroxymethyl furanone, also was demonstrated following an acute oral intake in a randomized, double-blind, placebo-controlled, 4-way crossover study involving (Moyad *et al.*, 2008). Fifteen (15) relatively healthy men were given 1 tablet of calcium ascorbate in a dose equivalent to 1,000 mg of ascorbic acid containing 1 or 3% calcium threonate, 1,000 mg of ascorbic acid, or placebo. None of the subjects dropped out of the study and no significant adverse effects were reported by subjects during the course of the study. Collectively, the available human data on Ester-C® and other calcium ascorbate preparations indicate that human consumption of Ester-C® at the intended use levels is not expected to be associated with any adverse effects.

The safety of ascorbic acid and/or calcium ascorbate has been reviewed by several recognized scientific committees, including JECFA, the Food and Nutrition Board of the IOM, the EFSA, and the United Kingdom's Expert Group on Vitamins and Minerals (EVM). In their safety evaluation of ascorbic acid and its calcium, potassium, and sodium salts for use as food additives and as vitamin C supplements, JECFA commented that animal studies demonstrated that ascorbic acid is not toxic after single or repeated administration of large doses (up to 2,500 mg/kg body weight/day in repeat-dose studies) (JECFA, 1981). The EFSA's Scientific Panel on Dietetic Products, Nutrition, and Allergies also concluded that ascorbic acid possesses low acute toxicity in animals and humans (EFSA, 2004). The IOM further noted that there is no evidence suggesting that ascorbic acid is carcinogenic or teratogenic or causes adverse reproductive effects (IOM, 2000). JECFA commented that in humans, daily doses of 100 to 6,000 mg have been taken over short periods of time with no adverse effects. Both the IOM and the EFSA's Scientific Panel concluded, however, that human ingestion of large doses of supplemental ascorbic acid, in the order of 3,000 mg or greater, was associated with acute gastrointestinal intolerances. Such gastrointestinal effects include flatulence, diarrhea, nausea, abdominal distention, and abdominal cramps and are attributed to the osmotic effect of unabsorbed ascorbic acid passing through the gastrointestinal tract. The EFSA's Scientific Panel commented though that limited data were available on the dose-response relationship for

these gastrointestinal effects and that very few controlled studies have been conducted to investigate the adverse effects of high-dose ascorbic acid intake in humans. With regards to other suspected adverse effects, the toxicity data does not provide sufficient evidence or support for a causal relationship between ascorbic acid intake by healthy individuals and other effects (IOM, 2000; EVM, 2003a; EFSA, 2004). The safety of long-term use of high-dose vitamin C supplements in humans has not been evaluated (EFSA, 2004).

Based on their evaluation, JECFA allotted an Acceptable Daily Intake (ADI) of "not specified[2]" for ascorbic acid and its calcium, potassium, and sodium salts. The IOM and EVM (EVM, 2003a) independently derived a lowest-observed-adverse-effect level (LOAEL) of 3,000 mg/person/day based on gastrointestinal disturbances observed following large bolus doses of supplemental vitamin C (and not following smaller vitamin C intakes from food ingested throughout the day). The IOM established the following ULs for vitamin C based on the LOAEL (also summarized in Table 2), and are applicable to intake from both food and supplements: 2,000 mg/person/day for adults aged 19 years and older; 400, 650, and 1,200 mg/person/day for children aged 1 to 3 years, 4 to 8 years, and 9 to 13 years, respectively; and 1,800 mg/person/day for adolescents and pregnant or lactating women. The IOM was not able to establish a UL for infants due to insufficient data on the adverse effects of vitamin C in this age group.

[2] A term applicable to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological, and other), the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food does not, in the opinion of JECFA, represent a hazard to health (WHO, 1987).

Table 2 Tolerable Upper Intake Levels (UL) and the Estimated Daily Intake of Vitamin C from All Intended Food Uses of Ester-C® in the U.S. by Population Group				
Population Group	**Age Group (Years)**	**UL (mg/day)**	**All-User Consumption**	
			Mean (mg)	**90th Percentile (mg)**
Infants and Young Children	0 to 12 months	Not applicable	302	642
	1 to 2	400		
Children	3	400	297	643
	4 to 8	650		
	9 to 11	1,200		
Female Teenagers	12 to 13	1,200	287	583
	14 to 18	1,800		
	19	2,000		
Male Teenagers	12 to 13	1,200	369	806
	14 to 18	1,800		
	19	2,000		
Female Adults	20 and Up	2,000	252	542
Male Adults	20 and Up	2,000	300	676
Total Population	All Ages	Not applicable	286	620

In contrast, the EFSA's Scientific Panel and the EVM determined that there were insufficient data to establish a UL for vitamin C from ascorbic acid and its calcium, potassium, and sodium salts; however, both concluded that supplemental daily doses of vitamin C up to approximately 1,000 mg/person in addition to vitamin C intake from the diet was not associated with adverse gastrointestinal effects, and that such intakes did not represent a cause for concern. The IOM also noted that the effects on which the UL is based are generally not serious and are self-limiting in that reducing supplemental vitamin C intakes will eliminate these effects. Furthermore, the EVM did not propose a guidance level, since adverse gastrointestinal effects are associated with supplemental, bolus doses of vitamin C rather than vitamin C intake from food. Therefore, Ester-C, when consumed as a component of food, would not be expected to result in any gastrointestinal adverse effects at the all-user total U.S. population 90th percentile estimated daily intakes of 810 mg/person/day providing vitamin C intakes of 620 mg/person/day, especially as such intakes are cumulative of smaller doses occurring throughout the course of the day. On an individual population basis, the all-user 90th percentile estimated daily intakes of vitamin C from the intended conditions of use of Ester-C® remain below the UL set by the IOM for each population group, except for young children ages 1 to 3 and children ages 4 to 8. To note, the ULs for children were extrapolated from that established for adults based on body weight differences. This methodology was undertaken by the IOM since the data on adverse effects in children were consistent with those in adults following bolus doses of supplemental vitamin C. These findings were supported by the results of a recent study conducted in children

aged 2 to 16 years in which the safety of vitamin C at levels near the respective ULs was demonstrated following two daily bolus supplemental intakes (Burns *et al.*, 2009).

In comparison to the form of vitamin C intake employed in human studies, the estimated daily intakes of vitamin C from the intended conditions of use of Ester-C® are divided doses taken with food and/or drink throughout the course of a day. It is not expected then that such intakes of vitamin C would cause the osmotic diarrhea or related gastrointestinal disturbances on which the ULs are based, since these effects are observed only following intake of large bolus doses of supplemental vitamin C. Indeed, the average dose of vitamin C from Ester-C intake that would be realistically ingested by infants and children per eating occasion was estimated to be less than 250 mg of vitamin C. This estimate was calculated based on the determination that children consume less than one serving size of 240 mL per eating occasion of the greatest contributors to Ester-C intake, which were fruit juices and fruit-flavored drinks and ades. Therefore, vitamin C consumption as a result of Ester-C® use in fruit juices and fruit-flavored drinks and ades is not a safety concern as it would be well below the level of intake necessary to produce osmotic diarrhea or other gastrointestinal side effects in children.

Briefly, with regards to safe levels of calcium intake, the IOM has set a UL of 2,500 mg/person/day from all sources (*e.g.*, food and supplements) for all age groups, including pregnant or lactating women, based on the risk for hypercalcemia and renal insufficiency at intakes ranging from 4,000 to 5,000 mg of calcium/person/day and greater (IOM, 1997). A UL for infants aged 0 to 12 months could not be established due to insufficient data. Likewise, the SCF established a UL of 2,500 mg/person/day from all sources for all age groups, including pregnant or lactating women, based on no adverse effects observed at this intake level in human studies (SCF, 2003). A UL could not be derived for infants, children, and adolescents based on insufficient data. The EVM determined that a UL for calcium could not be established based on insufficient data from animal and human studies; however, the EVM proposed that, for guidance purposes only, supplemental calcium intake of doses up to 1,500 mg/person/day would not be expected to result in adverse effects (EVM, 2003b). Established ULs and guidance levels for calcium are well in excess of the levels (all-user 90th percentile estimated daily intake of 74 mg/person/day) provided from the intended use levels of Ester-C®.

For calcium L-threonate, the EFSA's Scientific Panel on Food Additives and Nutrient Sources added to Food noted that calcium L-threonate has low oral acute toxicity, and from subchronic toxicity studies, identified a NOAEL of 4,000 and 1,000 mg/kg body weight/day in rats and dogs, respectively (EFSA, 2008). The Panel also noted that calcium L-threonate was not genotoxic, and that carcinogenicity studies were not required considering that L-threonate is physiologically present in the human body. Reproductive and developmental toxicity studies reviewed demonstrated no maternal or reproductive toxicity or teratogenicity in mice. The Panel concluded that the use of calcium L-threonate in food supplements is not of safety concern at levels providing 1,350 to 2,700 mg of L-threonate (L-threonic acid) per person per day, or

22.5 to 45 mg/kg body weight/day. These levels far exceed those (all-user 90th percentile estimated daily intake of 9 mg/person/day) provided from the intended use levels of Ester-C®. In addition, the EFSA's Scientific Panel on Food Additives, Flavorings, Processing Aids, and Materials in Contact with Food specifically evaluated the safety of calcium ascorbate with a content of threonate (*i.e.*, Ester-C® containing calcium threonate) as a source of ascorbic acid for use in food supplements (EFSA, 2007). Following review of the toxicological data, the Panel concluded that the additional exposure to calcium and threonate as a result of use of the ingredient is not of safety concern, and that the intended use levels of calcium ascorbate in the range of 646 to 1,292 mg (providing 500 to 1,000 mg of ascorbic acid), containing up to 2% threonate, as a source of ascorbic acid in food supplements is further not of safety concern. The conclusion of these safety assessments and the studies described above support the safe use of calcium L-threonate as a starting material in the manufacture of Ester-C® and as a component of the Ester-C® ingredient.

JECFA evaluated the safety of hydroxymethyl furanone and concluded that hydroxymethyl furanone does not present a safety concern at current levels of intake when used as a flavoring agent and has specified an ADI of "acceptable[3]" for this compound (JECFA, 2004). Following review of the available data presented in the toxicological monograph and given the low intakes of these flavoring agents in the European and U.S. populations, the Committee concluded that although shown to be genotoxic *in vitro*, it is highly unlikely that tetrahydrofuran or furanone derivatives would pose any significant genotoxic risk to humans under the conditions of use as flavoring agents (JECFA, 2006). Additionally, Ester-C® containing hydroxymethyl furanone (and calcium threonate) was demonstrated to be non-mutagenic as discussed above, indicating that hydroxymethyl furanone does not harbor mutagenic properties as it occurs in the Ester-C® ingredient. Furthermore, although the estimated intakes of hydroxymethyl furanone derived from the consumption of Ester-C®-containing foods are greater than current levels of intake as reported by JECFA (2006) (56 and 0.07 µg/person/day, or 0.9 and 0.001 µg/kg body weight/day, for the European and U.S. populations, respectively), subchronic and chronic studies demonstrated that hydroxymethyl furanone was not associated with any compound-related adverse or carcinogenic effects at doses up to 146 mg/kg body weight/day in rats. Therefore, a large margin of safety remains given that the estimated intakes of hydroxymethyl furanone from Ester-C®-containing foods are considerably low (all-user 90th percentile estimated daily intake of 364 µg/person/day).

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[3] A term used to describe flavouring agents that are of no safety concern at current levels of intake. If an ADI has been allocated to the agent, it is maintained unless otherwise indicated (WHO, 1987).

CONCLUSION

We, the Expert Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that the intended uses of Ester-C® Calcium Ascorbate (Ester-C®) meeting appropriate food-grade specifications presented in the supporting dossier [Documentation Supporting the Generally Recognized as Safe (GRAS) Status Ester-C® Calcium Ascorbate for Use In Traditional Food Products] and produced consistent with current good manufacturing practices (GMP), are safe and suitable.

We further conclude that the intended uses of Ester-C® Calcium Ascorbate, meeting appropriate food-grade specifications presented in the supporting dossier and produced consistent with current GMP, are GRAS based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

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Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Section Title
182—Substances generally recognized as safe	182.3013	Subpart D—Chemical Preservatives—Ascorbic acid
	182.8013	Subpart I—Nutrients—Ascorbic acid
184—Direct food substances affirmed as generally recognized as safe	184.1191	Calcium carbonate

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Ester-C®
The Ester C Company

November 6, 2012

Moraima Ramos-Valle, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Dear Dr. Ramos-Valle:

As requested by the United States Food and Drug Administration (FDA), please see the following supplementary information pertaining to the Ester-C Company's GRAS Notification of calcium ascorbate with a content of threonate (Ester-C®).

1. Calcium ascorbate with a content of threonate (Ester-C), as manufactured by the Ester-C company is intended for use as a dietary source of the calcium salts of ascorbic acid and threonic acid. This ingredient differs from food sources of vitamin C that are currently permitted for use in food (*i.e.*, ascorbic acid and calcium ascorbate) in the United States on the basis of its content of threonate. Ester-C notes that the presence of calcium threonate within Ester-C occurs as an intended product of the manufacturing conditions, whereby small quantities of ascorbic acid are oxidatively decomposed to normal metabolites of ascorbic acid, which include threonic acid. The content of threonic acid within the material is then standardized to a desired level of 1.15 ± 0.30% through the direct addition of threonic acid during manufacturing. The Ester-C company also notes the conditions of the manufacturing process also result in the production of small concentrations of hydroxymethylfuranone, a common flavoring agent, within the ingredient. These material differences in the ingredient composition of Ester-C relative to dietary sources of ascorbic acid that are regulated for use in food in the United States were considered by the Ester-C company to be sufficient to necessitate the conduct of a GRAS self-determination of the material.

Other regulatory differences between Ester-C and forms of vitamin C that are currently permitted for addition to food are noted. Under 21 CFR §182.3013 (U.S. FDA, 2012), the use of calcium ascorbate in food is limited by regulation to food uses as a preservative, and such uses do not extend to nutritive uses of the compound. Regulations or provisions authorizing the use of calcium ascorbate as a nutrient in food in the United States do not exist. Accordingly the intended food uses of Ester-C, as Notified to the FDA, have been the subject of a GRAS self-determination by the Ester-C company.

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2. The consumption of vitamin C with a content of threonate (Ester-C) is reported to improve vitamin C status in healthy subjects and animals (Verlangieri *et al.*, 1991; Moyad *et al.*, 2008, 2009). In a double-blind cross-over study conducted in 15 healthy male subjects (aged 18 to 50 years), the consumption of 1000 mg of vitamin C containing 1% threonate (Ester C) was reported to increase leukocyte vitamin C retention by 33% at 24 hours relative to concentrations observed when these same subjects consumed an equivalent dose of vitamin C (112.4±14.1 *vs.* 84.2±11.4 nmol/10^8 cells respectively; $p<0.001$). No differences in peak leukocyte vitamin C concentrations and no difference in plasma vitamin C concentrations were observed between treatments. In a second study, 50 healthy male and female subjects (39 female, 11 males; mean age 44 years; mean body weight 75 kg) were administered vitamin C with a content of threonate (Ester-C) or vitamin C in escalating doses (1000 mg on days 1 through 5 and 2000 mg on days 6 through 10) in a double-blind cross-over design. The authors reported that "*Mean WBC [white blood cell] vitamin C concentrations increased significantly over all periods for both treatments but was only highly significant (P<0.001) over all time intervals for vitamin C with metabolites*". No differences in plasma vitamin C content were observed between treatment allocations. Finally, administration of Ester-C to Osteogenic Disorder Shionogi (ODS) rats is reported to improve vitamin C status at the cellular level and produce improved functional outcomes in scorbutic rats relative to that observed with equivalent doses of vitamin C (Verlangieri *et al.*, 1991). The FDA has requested additional information pertinent to the safety of these reported findings among frequent consumers of Ester-C containing foods.

The nutritional role of vitamin C is based entirely on its capacity to serve as a reducing equivalent for a variety of biological functions (IOM, 2000). Vitamin C serves as an electron donor for 8 human enzymes that mediate collagen hydroxylation, carnitine biosynthesis, and hormone and amino acid biosynthesis. Vitamin C is an important nutrient required for normal immune function. The reducing capacity of vitamin C is necessary for optimal microbicidal activities of leukocytes, and is involved in lymphocyte proliferation and chemotaxis (IOM, 2000). Blood levels of vitamin C in healthy individuals are approximately 50 µmol/L (Vera *et al.*, 1998); however leukocyte concentrations of vitamin C are reported to be a more accurate reflection of whole body vitamin C status than plasma or erythrocyte concentrations (IOM, 2000). Concentrations of vitamin C within leukocytes typically exceed concentrations present within the blood by orders of magnitude, and intracellular concentrations within the mmol/L range is normal (Vera *et al.*, 1998). The high intracellular concentrations of vitamin C within leukocytes protect against oxidant damage from reactive oxygen species generated during respiratory burst, and serve to neutralize phagocyte derived oxidants without inhibiting the bactericidal activity of the phagosome (IOM, 2000). Accumulation of vitamin C within leukocytes is therefore a normal and nutritionally beneficial response to increased plasma concentrations of the vitamin. The baseline leukocyte concentrations of vitamin C in healthy individuals are highly variable as they are influenced by dietary intake of the vitamin; concentrations of vitamin C between 5 to 835 nmol/10^8 cells have been reported across a number of studies in healthy adult subjects (Jacob, 1990). These concentrations are well within the concentration ranges that have been reported among individuals consuming Ester-C (1000 to 2000 mg/day) in controlled settings (Moyad *et al.*, 2008, 2009). Based on the antioxidant role of vitamin C in human nutrition, abnormally high intracellular concentrations of vitamin C could, in theory, produce adverse effects associated with extensive superoxide/H_2O_2 formation, and these effects have been reported to occur in cell cultures incubated with supraphysiologic (mmol/L) concentrations

of vitamin C (Azzolini *et al.*, 2012). However, as reported by Moyad *et al.* (2008), intracellular concentrations of vitamin C in subjects administered Ester-C (112.4±14.1 mmol/108 cells) were only increased by ~30% above those observed following vitamin C consumption at the 24-hour time-point. This increase is well within the normal baseline range that is seen within leukocytes in healthy humans and therefore would not be expected to be of adverse consequence. This conclusion is consistent with *in vitro* findings where incubation of human myeloma cells (U937) with 30 µmol/L ascorbic acid, producing estimated intracellular concentrations of 127 nmol vitamin C/10^8 cells/L[1] was not associated with changes in the redox state of the cells: intracellular concentrations of GSH, NADH, NAD^+, NAD(P)H, and $NADP^+$ remained unchanged (Azzolini *et al.*, 2012).

The mechanism(s) for the improvement in vitamin C status among subjects consuming Ester-C as reported by Moyad *et al.* (2008, 2009) are unknown; however, the important biological role of oxidized vitamin C in immune cell function is well established. *In vivo* production of oxidized vitamin C in aerobic organisms is a normal occurrence, and small amounts of vitamin C are continually lost through oxidative catabolism, particularly within sites where immune mediated oxidative effects occur. The primary products of vitamin C oxidation include dehydroascorbic acid, oxalic and threonic acids, L-xylose, and ascorbate 2-sulphate (IOM, 2000). The oxidative metabolite dehydroascorbic is widely recognized for its participation in the phenomenon of vitamin C recycling, a process whereby dehydroascorbate, produced by oxidation of extracellular vitamin C, is transported into the leukocyte *via* facilitative glucose transporters and is then enzymatically reduced back to vitamin C such that high intracellular concentrations of the vitamin can be maintained (Nualart et al., 2003). *In vitro* findings have suggested that the transport of dehydroascorbic acid through facilitative glucose transporters followed by enzymatic reduction to vitamin C within the cell may be the predominant mechanism by which host defense cells acquire vitamin C under oxidative conditions (Vera *et al.*, 1998; Corpe *et al.*, 2005). There is limited information on the potential role of other vitamin C metabolites on immune function; however, other metabolites such as threonic acid may have nutritional roles in maintaining optimal vitamin C status humans. For example, incubation of human T-lymphoma cell lines with radiolabeled vitamin C containing increasing concentrations of calcium threonate is reported to increase intracellular levels of vitamin C above that observed with vitamin C alone (Fay and Verlangieri, 1991).

Taken together it can be concluded that the improvements in leukocyte concentrations of vitamin C observed in controlled settings in healthy subjects administered Ester-C are normal and nutritionally desirable changes that are representative of optimal vitamin C status. Production of vitamin C metabolites through oxidative processes is expected to occur on a normal basis in humans, and oxidized forms of vitamin C metabolites (*i.e.*, dehydroascorbic acid) are necessary for optimal functioning of the immune system. Additional corroborative evidence supporting the safety of exposure to vitamin C metabolites within Ester-C is supported by the absence of adverse clinical chemistry, hematological, or histopathological findings following the sub-acute administration of Ester-C to Sprague-Dawley rats at a dose as high as 8,744 mg Ester-C/kg body weight per day for 28 days (see Section 2.1 of Ester-C's GRAS Notification). Similarly, administration of threonate in the diet at a concentration of 1.0% to

[1] Calculated from reported intracellular concentration of 0.25 mmol/L and average leukocyte volume of 500 cubic microns (Tivey *et al.*, 1951).

young male Wistar rats for 120 days was non-toxic, and chronic administration of threonate in the diet of 5-week-old mice (Tuck No. 1 strain) at a concentration of up to 0.2% for the lifetime of the animals was without evidence of toxicity (Thomas and Hughes, 1985). Unlike humans, rodents are capable of synthesizing vitamin C endogenously; therefore potential adverse interactions/interferences of threonate on vitamin C metabolism are expected to have been detected in these studies. Finally, the findings by Moyad *et al.* (2009) were reviewed by the Expert Panel who concluded that these studies were not pivotal to the safety assessment of Ester-C.

We thank you for the opportunity to provide the Agency with additional information supporting the safety of Ester-C and the Ester-C company is happy to provide additional data and information as needed.

Sincerely,

(b) (6)

Maile Combs, MS
Assoc Dir, Scientific Affairs
The Ester C Company

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I GRAS Exemption Claim

I.A Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)] (U.S. FDA, 1997)

The Ester C Company hereby claims that the use of Ester-C® [calcium ascorbate with added threonate] as a nutrient in foods, as described in Section I.D below, is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because we have determined that such use are Generally Recognized as Safe (GRAS).

Signed,

(b) (6)

11/14/12

Maile Combs MS
Assoc. Director Scientific Affairs
The Ester C Company

Date

I.B Name and Address of Notifier

The Ester C Company
6735 Inter-Cal Way
Prescott, Arizona 86301
USA
Tel: 928-541-2269
Fax: 928-777-2459

I.C Common Name of the Notified Substance

Calcium ascorbate with threonate

I.D Conditions of Intended Use in Food

1. Foods in Which the Substance is to be Used

The Ester C Company intends to use Ester-C® as a nutrient in conventional foods and beverages. The individual intended food uses, maximum use-levels for Ester-C®, and corresponding maximum use-levels of ascorbic acid (vitamin C), are summarized in Table I.D-1. In those foods and beverages already fortified with a source of vitamin C, Ester-C® is intended to be added in replacement of, and not in addition to, the existing vitamin C fortificant. In addition to the traditional food uses listed in Table I.D-1, the Ester C Company also intends to

use Ester-C® as a nutrient in medical foods at use-levels providing up to 500 mg of vitamin C per serving.

Table I.D-1 Summary of the Individual Intended Food Uses and Use-levels for Ester-C® and the Corresponding Use-levels of Vitamin C in the U.S.

Food Category	Intended Food Uses	RACC (g/mL)[a]	Ester-C®		Vitamin C	
			Use level[b] (mg/ serving)	Use level (%)	Level (mg/ serving)	Level (%)
Beverages and Beverage Bases	Energy Drinks	240	326	0.14	250	0.10
	Fruit-Flavored Drinks and Ades (with Vitamin C Added)	240	326	0.14	250	0.10
	Meal Replacement Beverages (Powdered Only)	240	326	0.14	250	0.10
Breakfast Cereals	Instant Oatmeal	240	78	0.033	60	0.025
	RTE Breakfast Cereals[c]	15 (Puffed) 30 (Regular) 55 (Biscuit-Type)	78	0.52 0.26 0.14	60	0.40 0.20 0.11
Chewing Gum	Chewing Gum	3	163	5.44	125	4.17
Coffee and Tea	RTD Teas (Presweetened, Not Powdered)	240	163	0.068	125	0.052
Grain Products	Breakfast and Meal Replacement Bars	40	163	0.41	125	0.31
Processed Fruits and Fruit Juices	Fruit Juice (Excluding Fruit Juice Blends)[c]	240	326	0.14	250	0.10
Medical Foods	Medical Foods	N/A	653	N/A	500	N/A

N/A = not applicable; RTD = Ready-to-drink; RTE = Ready-to-eat
[a] RACC = Reference Amounts Customarily Consumed per Eating Occasion (21 CFR §101.12 – U.S. FDA, 2012a). When a range of values is reported for an intended food use, particular foods within that food use may differ with respect to their RACC.
[b] Use level of Ester-C® = Use level of Vitamin C/0.766
[c] The food codes listed under this food use were selected by The Ester C Company.

2. Purpose for Which Substance is Used

Ester C is intended to be used in foods as a dietary source of calcium ascorbate and calcium threonate[1].

[1] Although ascorbic acid is a GRAS substance when used as a nutrient (21 CFR §182.8013) or chemical preservative (21 CFR §182.3013) in accordance with good manufacturing practices (GMP), these provisions do not extend to the calcium salt of ascorbic acid. Under 21CFR§182.3189 calcium ascorbate is generally recognized as safe when used as a preservative in accordance with good manufacturing practice; this use does not extend to food uses as a nutrient. Various calcium salts are GRAS or are affirmed as GRAS by the FDA as direct food substances; however, provisions permitting the addition of calcium L-threonate to food as a direct food ingredient do not exist.

3. Description of the Population Expected to Consume the Substance

Ester C is expected to be consumed by members of the general population who may be reasonably be expected to consume at least one food within the food categories described above.

I.E Basis for the GRAS Determination

Pursuant to Title 21, Section 170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2012b), Ester-C® [calcium ascorbate with threonate] has been determined to be GRAS on the basis of scientific procedures.

I.F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

The Ester C Company
6735 Inter-Cal Way
Prescott, Arizona 86301
USA

Should the FDA have any questions or additional information requests regarding this notification, the Ester C Company will supply these data and information.

II Detailed Information about the Identity of the Notified Substance

II.A Identity

Ester-C® is a tan, free flowing powder with a slight caramel odor and is characterized as comprising 95.5% calcium ascorbate dihydrate, 1.2% calcium threonate, 1.1% calcium carbonate and not more than 2.0% moisture by weight. Ester-C® is free of genetically modified organisms, contains no materials of animal origin, is free from artificial colors, flavors, and preservatives, is not irradiated or sterilized in any way, and does not contain any carriers.

Common or Usual Name:	Calcium ascorbate
Chemical Name:	Calcium ascorbate dihydrate
Chemical Abstracts Service (CAS) Number:	201542-81-6
Empirical Formula and Formula Weight:	$C_{12}H_{14}CaO_{12}\cdot 2H_2O$

Ester-C
The Ester C Company

November 6, 2012

Moraima Ramos-Valle, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Dear Dr. Ramos-Valle:

As requested by the United States Food and Drug Administration (FDA), please see the following supplementary information pertaining to the Ester-C Company's GRAS Notification of calcium ascorbate with a content of threonate (Ester-C®).

1. Calcium ascorbate with a content of threonate (Ester-C), as manufactured by the Ester-C company is intended for use as a dietary source of the calcium salts of ascorbic acid and threonic acid. This ingredient differs from food sources of vitamin C that are currently permitted for use in food (*i.e.*, ascorbic acid and calcium ascorbate) in the United States on the basis of its content of threonate. Ester-C notes that the presence of calcium threonate within Ester-C occurs as an intended product of the manufacturing conditions, whereby small quantities of ascorbic acid are oxidatively decomposed to normal metabolites of ascorbic acid, which include threonic acid. The content of threonic acid within the material is then standardized to a desired level of 1.15 ± 0.30% through the direct addition of threonic acid during manufacturing. The Ester-C company also notes the conditions of the manufacturing process also result in the production of small concentrations of hydroxymethylfuranone, a common flavoring agent, within the ingredient. These material differences in the ingredient composition of Ester-C relative to dietary sources of ascorbic acid that are regulated for use in food in the United States were considered by the Ester-C company to be sufficient to necessitate the conduct of a GRAS self-determination of the material.

Other regulatory differences between Ester-C and forms of vitamin C that are currently permitted for addition to food are noted. Under 21 CFR §182.3013 (U.S. FDA, 2012), the use of calcium ascorbate in food is limited by regulation to food uses as a preservative, and such uses do not extend to nutritive uses of the compound. Regulations or provisions authorizing the use of calcium ascorbate as a nutrient in food in the United States do not exist. Accordingly the intended food uses of Ester-C, as Notified to the FDA, have been the subject of a GRAS self-determination by the Ester-C company.

2. The consumption of vitamin C with a content of threonate (Ester-C) is reported to improve vitamin C status in healthy subjects and animals (Verlangieri *et al.*, 1991; Moyad *et al.*, 2008, 2009). In a double-blind cross-over study conducted in 15 healthy male subjects (aged 18 to 50

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years), the consumption of 1000 mg of vitamin C containing 1% threonate (Ester C) was reported to increase leukocyte vitamin C retention by 33% at 24 hours relative to concentrations observed when these same subjects consumed an equivalent dose of vitamin C (112.4±14.1 *vs.* 84.2±11.4 nmol/10^8 cells respectively; $p<0.001$). No differences in peak leukocyte vitamin C concentrations and no difference in plasma vitamin C concentrations were observed between treatments. In a second study, 50 healthy male and female subjects (39 female, 11 males; mean age 44 years; mean body weight 75 kg) were administered vitamin C with a content of threonate (Ester-C) or vitamin C in escalating doses (1000 mg on days 1 through 5 and 2000 mg on days 6 through 10) in a double-blind cross-over design. The authors reported that "*Mean WBC [white blood cell] vitamin C concentrations increased significantly over all periods for both treatments but was only highly significant (P<0.001) over all time intervals for vitamin C with metabolites*". No differences in plasma vitamin C content were observed between treatment allocations. Finally, administration of Ester-C to Osteogenic Disorder Shionogi (ODS) rats is reported to improve vitamin C status at the cellular level and produce improved functional outcomes in scorbutic rats relative to that observed with equivalent doses of vitamin C (Verlangieri *et al.*, 1991). The FDA has requested additional information pertinent to the safety of these reported findings among frequent consumers of Ester-C containing foods.

The nutritional role of vitamin C is based entirely on its capacity to serve as a reducing equivalent for a variety of biological functions (IOM, 2000). Vitamin C serves as an electron donor for 8 human enzymes that mediate collagen hydroxylation, carnitine biosynthesis, and hormone and amino acid biosynthesis. Vitamin C is an important nutrient required for normal immune function. The reducing capacity of vitamin C is necessary for optimal microbicidal activities of leukocytes, and is involved in lymphocyte proliferation and chemotaxis (IOM, 2000). Blood levels of vitamin C in healthy individuals are approximately 50 µmol/L (Vera *et al.*, 1998); however leukocyte concentrations of vitamin C are reported to be a more accurate reflection of whole body vitamin C status than plasma or erythrocyte concentrations (IOM, 2000). Concentrations of vitamin C within leukocytes typically exceed concentrations present within the blood by orders of magnitude, and intracellular concentrations within the mmol/L range is normal (Vera *et al.*, 1998). The high intracellular concentrations of vitamin C within leukocytes protect against oxidant damage from reactive oxygen species generated during respiratory burst, and serve to neutralize phagocyte derived oxidants without inhibiting the bactericidal activity of the phagosome (IOM, 2000). Accumulation of vitamin C within leukocytes is therefore a normal and nutritionally beneficial response to increased plasma concentrations of the vitamin. The baseline leukocyte concentrations of vitamin C in healthy individuals are highly variable as they are influenced by dietary intake of the vitamin; concentrations of vitamin C between 5 to 835 nmol/10^8 cells have been reported across a number of studies in healthy adult subjects (Jacob, 1990). These concentrations are well within the concentration ranges that have been reported among individuals consuming Ester-C (1000 to 2000 mg/day) in controlled settings (Moyad *et al.*, 2008, 2009). Based on the antioxidant role of vitamin C in human nutrition, abnormally high intracellular concentrations of vitamin C could, in theory, produce adverse effects associated with extensive superoxide/H_2O_2 formation, and these effects have been reported to occur in cell cultures incubated with supraphysiologic (mmol/L) concentrations of vitamin C (Azzolini *et al.*, 2012). However, as reported by Moyad *et al.* (2008), intracellular

concentrations of vitamin C in subjects administered Ester-C (112.4±14.1 nmol/10^8 cells[1]) were only increased by ~30% above those observed following vitamin C consumption at the 24-hour time-point. This increase is well within the normal baseline range that is seen within leukocytes in healthy humans and therefore would not be expected to be of adverse consequence. This conclusion is consistent with *in vitro* findings where incubation of human myeloma cells (U937) with 30 µmol/L ascorbic acid, producing estimated intracellular concentrations of 127 nmol vitamin C/10^8 cells/L[2] was not associated with changes in the redox state of the cells: intracellular concentrations of GSH, NADH, NAD^+, NAD(P)H, and $NADP^+$ remained unchanged (Azzolini *et al.*, 2012).

The mechanism(s) for the improvement in vitamin C status among subjects consuming Ester-C as reported by Moyad *et al.* (2008, 2009) are unknown; however, the important biological role of oxidized vitamin C in immune cell function is well established. *In vivo* production of oxidized vitamin C in aerobic organisms is a normal occurrence, and small amounts of vitamin C are continually lost through oxidative catabolism, particularly within sites where immune mediated oxidative effects occur. The primary products of vitamin C oxidation include dehydroascorbic acid, oxalic and threonic acids, L-xylose, and ascorbate 2-sulphate (IOM, 2000). The oxidative metabolite dehydroascorbic is widely recognized for its participation in the phenomenon of vitamin C recycling, a process whereby dehydroascorbate, produced by oxidation of extracellular vitamin C, is transported into the leukocyte *via* facilitative glucose transporters and is then enzymatically reduced back to vitamin C such that high intracellular concentrations of the vitamin can be maintained (Nualart et al., 2003). *In vitro* findings have suggested that the transport of dehydroascorbic acid through facilitative glucose transporters followed by enzymatic reduction to vitamin C within the cell may be the predominant mechanism by which host defense cells acquire vitamin C under oxidative conditions (Vera *et al.*, 1998; Corpe *et al.*, 2005). There is limited information on the potential role of other vitamin C metabolites on immune function; however, other metabolites such as threonic acid may have nutritional roles in maintaining optimal vitamin C status in humans. For example, incubation of human T-lymphoma cell lines with radiolabeled vitamin C containing increasing concentrations of calcium threonate is reported to increase intracellular levels of vitamin C above that observed with vitamin C alone (Fay and Verlangieri, 1991).

Taken together it can be concluded that the improvements in leukocyte concentrations of vitamin C observed in controlled settings in healthy subjects administered Ester-C are normal and nutritionally desirable changes that are representative of optimal vitamin C status. Production of vitamin C metabolites through oxidative processes is expected to occur on a normal basis in humans, and oxidized metabolites of vitamin C (*i.e.*, dehydroascorbic acid) are necessary for optimal functioning of the immune system. Additional corroborative evidence supporting the safety of exposure to vitamin C metabolites within Ester-C is supported by the absence of adverse clinical chemistry, hematological, or histopathological findings following the sub-acute administration of Ester-C to Sprague-Dawley rats at a dose as high as 8,744 mg Ester-C/kg body weight per day for 28 days (see Section 2.1 of Ester-C's GRAS Notification).

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[1] The y-axis unit (mmol/10^8 cells) reported by Moyad *et al.*, (2008) in figure 3 is incorrect as published. Verification of the original study data by the Ester-C Company confirmed that the correct unit is nmol/10^8 cells.

[2] Calculated from reported intracellular concentration of 0.25 mmol/L and average leukocyte volume of 500 cubic microns (Tivey *et al.*, 1951).

Similarly, administration of threonate in the diet at a concentration of 1.0% to young male Wistar rats for 120 days was non-toxic, and chronic administration of threonate in the diet of 5-week-old mice (Tuck No. 1 strain) at a concentration of up to 0.2% for the lifetime of the animals was without evidence of toxicity (Thomas and Hughes, 1985). Unlike humans, rodents are capable of synthesizing vitamin C endogenously; therefore potential adverse interactions/interferences of threonate on vitamin C metabolism are expected to have been detected in these studies. Finally, the findings by Moyad *et al.* (2009) were reviewed by the Expert Panel who concluded that these studies were not pivotal to the safety assessment of Ester-C.

We thank you for the opportunity to provide the Agency with additional information supporting the safety of Ester-C and the Ester-C company is happy to provide additional data and information as needed.

Sincerely,

(b) (6)

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